BINDING OF BILIRUBIN TO ERYTHROCYTES FROM DIFFERENT MAMMALIAN SPECIES

BY

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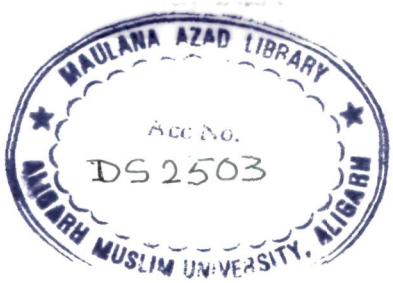

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CERTIFICATE

I certify that the work presented in the following pages has been carried out by Mr. Mohammad Kutub Ali and that it is suitable for the award of M.Phil. degree in Biotechnology of the Aligarh Muslim University, Aligarh.

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ABSTRACT

Although multisite toxicity of bilirubin towards metabolism of cells is well known (Karp, 1979), mechanism of bilirubin entry from plasma to cells is still an enigma. Several studies have been performed to unravel the pathway (s) through which bilirubin enters into the cells using red blood cells as a model. But there is no clear evidence about the nature of membrane receptors for bilirubin. However, participation of membrane proteins as receptors has been excluded in an earlier study, but it is suggested that membrane proteins may function as an effective barrier to the binding of bilirubin (Sato & Kashiwamata, 1983). Further, it is proposed that negatively charged phosphate groups of phospholipids on the membrane surface may prevent a large amount of bilirubin from binding to the membrane (Sato *et al.* 1987). Since the membrane make-up of erythrocytes varies from species to species (Lenard, 1970; Barenholz & Thompson, 1980), we have studied the binding of bilirubin to erythrocytes from four different species namely, human, goat, buffalo and sheep both in the presence as well as in the absence of their respective plasma albumins under identical conditions of pH (8.0), temperature (37°C) and ionic strength (0.414).

When the bilirubin concentration was kept constant and albumin concentration varied to give different bilirubin/albumin molar ratios in the range of 0.5 to 3.0, the amount of erythrocyte-bound bilirubin increased with the increase in bilirubin/albumin molar ratio. The increase was four fold at

bilirubin/albumin molar ratio of 2.0. The pattern of bilirubin binding to erythrocytes at increasing bilirubin/albumin molar ratios was found to be similar under both the conditions; i.e. at constant bilirubin and increasing albumin concentrations or at constant albumin and increasing bilirubin concentrations. However, at any given bilirubin/albumin molar ratio above 1:1 under both the above conditions, goat erythrocytes bound the highest amount of bilirubin followed by buffalo and human erythrocytes, whereas sheep erythrocytes bound the lowest amount of bilirubin. Further, at a given constant bilirubin/ albumin molar ratio, the binding of bilirubin to erythrocytes increased linearly with increase in bilirubin concentration. However, the value of the slope of the linear plots at any given bilirubin/albumin molar ratio was found to be different for different erythrocytes being highest for goat erythrocytes followed by buffalo and human erythrocytes and lowest for sheep erythrocytes. When slopes were plotted against bilirubin/albumin molar ratio, there was an initial rapid increase in the slope and then it became somewhat constant at higher molar ratios, suggesting the saturation of bilirubin binding sites in the erythrocyte membranes.

The percentage difference in fractional binding of bilirubin to erythrocytes as calculated from the above data was found highest in between bilirubin/ albumin molar ratios of 1.5 and 2.0. However, at other molar ratios, its value was nearly equal. A comparison of the percentage difference in fractional bilirubin binding at molar ratios 1.5 to 2.0 shows that goat erythrocytes have the highest percentage followed by buffalo and human erythrocytes and sheep

erythrocytes have the lowest value. Further, calculation of the relative percentage difference in the bilirubin bound to erythrocytes of sheep, buffalo and goat with respect to the bilirubin bound to human erythrocytes shows that goat and buffalo erythrocytes bind bilirubin 31.8% and 11.4% respectively more whereas sheep erythrocytes bind 8.7% less bilirubin. These differences in erythrocyte-bound bilirubin can not be attributed to the albumin effect as the elution of erythrocyte-bound bilirubin with human, goat, buffalo and sheep plasma albumins yielded the same amount of bilirubin from human erythrocytes. Further, in all the mammalian species studied, the binding of bilirubin to erythrocytes was found to be more or less completely protected in the presence of their respective albumins at a bilirubin/albumin molar ratio of 0.5. All these results, in combine, suggest that difference in bilirubin binding to various mammalian erythrocytes might be due to the difference in the number as well as the affinities of the receptors present on these membranes.

In the absence of albumin, curves between erythrocyte-bound bilirubin and bilirubin in the incubate followed Michaelian saturation kinetics. The dissociation constant of the bilirubin-receptor complex and saturation were calculated using double reciprocal plots. Among the various erythrocytes, goat erythrocytes had the highest dissociation constant (265.7 μmol/l) and highest saturation (125.9 μM) whereas sheep erythrocytes had the lowest dissociation constant (115.6 μmol/l) and lowest saturation (62.5 μM). Buffalo and human erythrocytes bound bilirubin in a similar fashion and the values of interaction parameters were in between the values obtained with those of goat and sheep

erythrocytes. Thus, it appears that the number of saturable bilirubin binding sites on goat erythrocytes is highest of all the species studied whereas the number of sites on sheep erythrocytes is the lowest. Human and buffalo erythrocytes have nearly the same number of binding sites and the values were in between the values obtained with goat and sheep erythrocytes. In contrast, the affinity of these sites is highest in sheep erythrocytes and lowest in goat erythrocytes. Human and buffalo erythrocytes have similar affinities falling in between the affinities or sheep and goat erythrocytes.

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Aligarh.

July

M. K. Ali

(Mohammad Kutub Ali)

Dedicated

to my Father

and

to the Memory

of my (late) Mother.

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INTRODUCTION

Under physiological conditions, in human adult, 1-2 x 10^8 erythrocytes are destroyed per hour resulting in a turn over of approximately 6 gm of haemoglobin per day by a 70 kg human (Schmid & McDonough, 1978). Both the protein, globin and iron of haemoglogin are reutilized by the body, but the iron-free porphyrin portion of haeme is catabolized mainly in the reticuloendothelial cells of the liver, spleen, and bone marrow producing a yellow pigment called bilirubin IX-α Bilirubin IX-α can be isolated from gall stones and is the most commonly found natural linear tetrapyrrole. It is estimated that 1 gm of haemoglobin yields 35 mg of bilirubin. Thus, about 250-350 mg of bilirubin is produced per day in human adults.

Properties of bilirubin

(i) Chemical properties : Bilirubin is described as a polar structure with several groups capable of forming hydrogen bonds, namely, two carboxyls, two pyrrol -NH, two lactam-NH and two lactam carbonyls and several hydrophobic groups i.e. four methyls, two vinyls, two ethylenes, and one methylene (see Fig.1 a & b). The protoporphyrin IX, the parent molecule, which occurs in Z- configuration mainly opens at the α- methin bridge during catabolism, leading to the formation of bilirubin IX-α (Z,Z). This is present either as dianion or intramolecularly hydrogen- bonded bilirubin acid as shown in Fig. 1 b. However, little amounts of protoporphyrin IX also undergo cleavage at the ß, γ or δ positions yielding about 5% of bilirubin non-α isomers

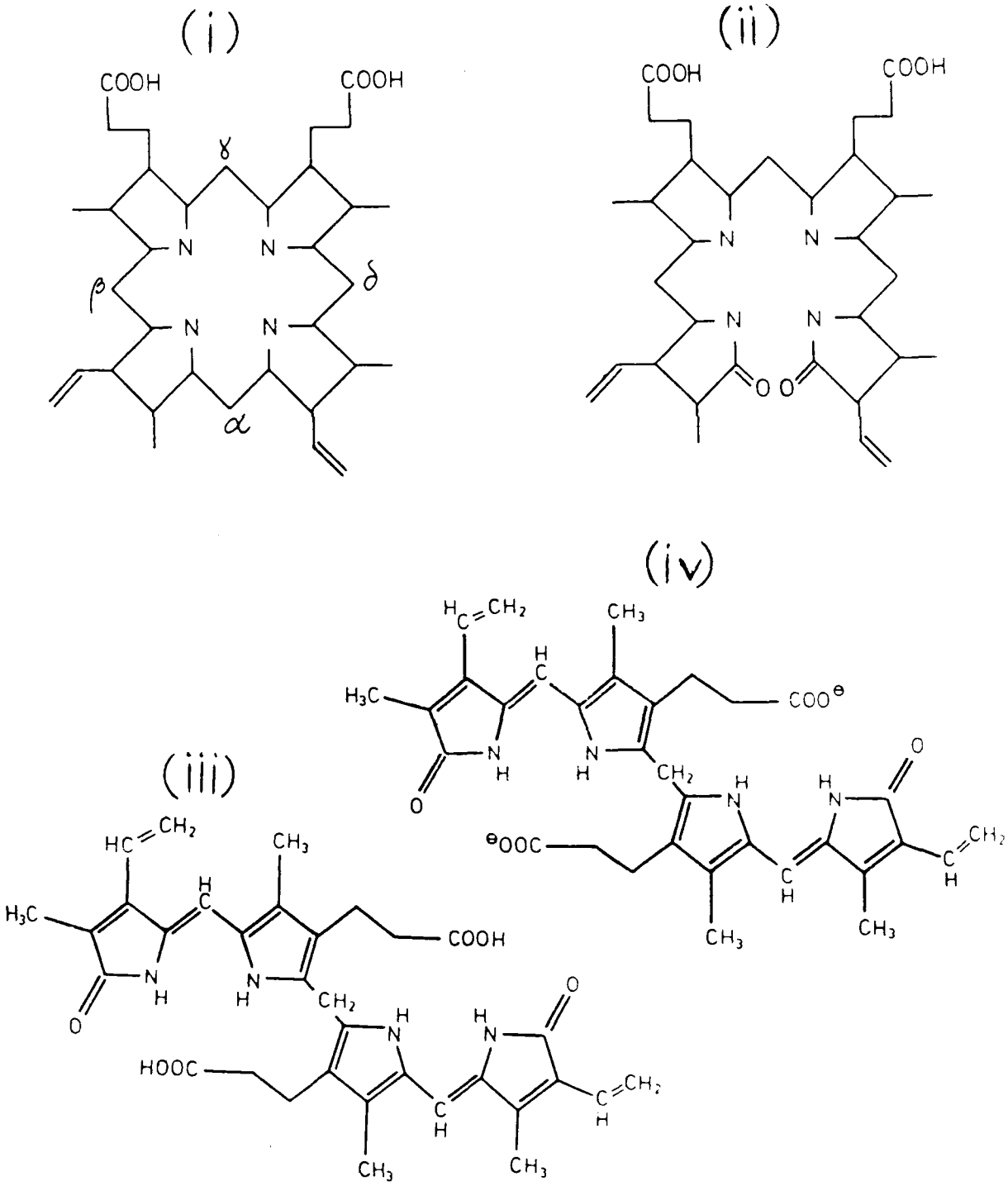

Figure 1. (a) Chemical structure of bilirubin.

(i) Protoporphyrin IX. (ii) Bilirubin IX-α. (iii) Bilirubin IX-α (acid). (iv) Bilirubin IX-α (dianion).

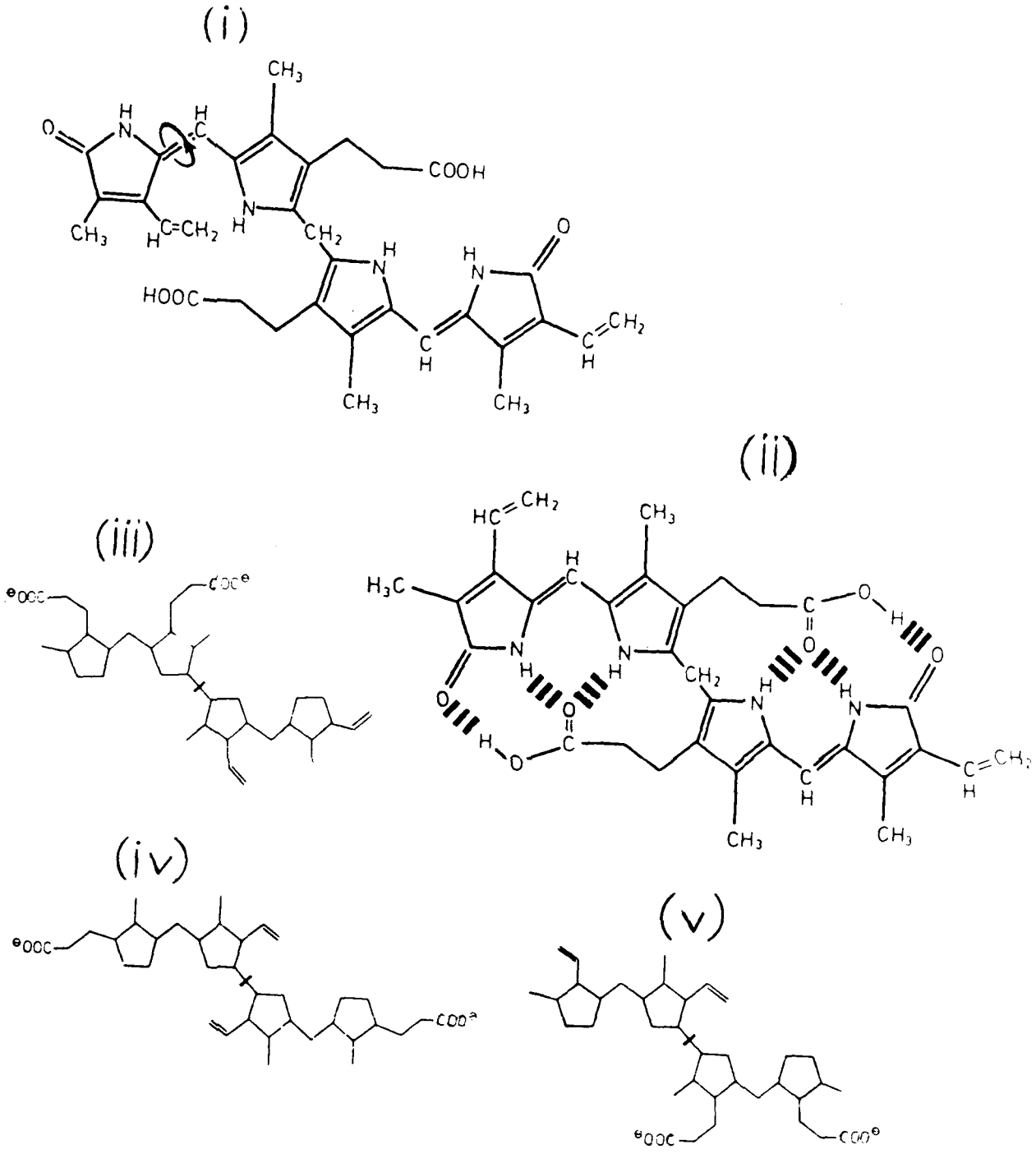

Figure 1. (b) Photoisomers and isomers of bilirubin.

(i) The mother compound, bilirubin IX-α (Z,Z) is transformed into (E,Z) or possibly (E,E), also called photobilirubins. (ii) Bilirubin IX-α (acid), intramolecularly hydrogen bonded (iii) Bilirubin IX-β. (iv) Bilirubin IX-γ. and (v) Bilirubin IX-δ

such as bilirubin IX-ß, IX-γ or IX-δ (Brown, 1976; Blanckaert *et al.*, 1975, 1976; Blumenthal *et al.*, 1977; Heirwegh *et al.*, 1977). Upon illumination with blue light, bilirubin IX-α (Z,Z) undergoes cis-trans isomerization producing bilirubin IX-α (E,Z) or possibly bilirubin IX-α (E,E) called photobilirubins (Lightner & Park, 1977, Pedersen *et al.*, 1977) which can no longer form the pattern of intramolecular hydrogen bonds.

(ii) Solubility : Bilirubin IX-α (Z,Z) is nearly insoluble in water at pH values below 7 while non-α isomers and the photobilirubins (E,Z and E,E) are more soluble (Overbeek *et al.*, 1955; Brodersen, 1979). Only α- isomers in (Z,Z) configuration, with undissociated carboxyl groups are capable of forming a complete pattern of intramolecular hydrogen bonds, saturating all affinities for water and are insoluble. In non-α isomers, the carboxyl groups are located differently, relative to the lactam groups and pyrrol nitrogen atoms and this is also the case in photoisomers where the outer rings have been turned, forming E-configurations. All these have one or several hydrophilic moieties available for binding of water molecules and thus are water soluble even in acid solution. The solubility of bilirubin acid being too low in water, has been measured in several solvents (Brodersen, 1979). The solubility of bilirubin in apolar solvents, such as n-hexane is less than 1 μM and generally increases with increasing solvent polarity, reaching to a value higher than 10 mM in dimethyl sulphoxide (Brodersen, 1979).

Several authors have stated that bilirubin is a lipophilic substance. This is mainly due to the work of Mustafa and King (1970) who demonstrated the

binding of bilirubin to lipid-water interphases containing lipids of a polar nature. Another consideration of bilirubin lipophilicity is the insolubility of bilirubin acid in water due to intramolecular hydrogen bonding (Bonnett *et al.*, 1976), wherby all hydrophilic groups in the molecule are saturated. This molecule is also insoluble in carbon hydrides and alcohols and has a low solubility in carbon tetrachloride, diethyl ether, acetone, ethyl acetate and triglycerides, while its solubility is high in formamide and dimethyl sulphoxide. A substance with these solubility characteristics can hardly be considered as lipophilic (Brodersen, 1979).

(iii) Spectroscopic properties : Light absorption spectrum of bilirubin IX-α (Z,Z) acid, dissolved in organic solvents shows two maxima, one weak band at about 290 nm and another intense maximum at 450 to 460 nm (Heilmeyer, 1931), while spectrum of non-α isomers of bilirubin in dimethyl-formamide solution shows similar maximum although the main peak is located at shorter wavelength 390 to 410 nm (Blanckaert *et al.*, 1976). Bilirubin IX-α (Z,Z) acid dissolved in chloroform has light absorption maximum at 454 nm and a specific extinction coefficient close to 1.04×10^3 (Jacobsen & Brodersen, 1983).

Dilute solutions of bilirubin disodium salt in alkaline aqueous media have light absorption maximum around 430 to 440 nm, depending upon buffer composition and temperature (Carey & Koretsky, 1979). A molar absorption coefficient of 4.7 to 5.2×10^4 M^{-1} cm^{-1} has been reported by several investigators (Carey & Koretsky, 1979; McDonagh, 1979; Blauer *et al.*, 1972).

The spectrum is constant with pH varying from 8 to 12. At low concentrations of bilirubin, from about 20 nM, which is the practical lower limit for spectroscopic recording, to an upper limit between 1.0 to 10 μM, the spectrum remains constant when normalized to one concentration. But if the alkaline bilirubin solution is fairly concentrated (0.1 mM or more) and is neutralized to pH 7.0, colloid aggregation of the acid can be observed by an immediate rise of light scattering to high values, indicating formation of large particles. The light absorption spectrum shows a slight, immediate loss of intensity followed by marked decrease of the peak at 440 nm and an increase of absorption at 500 nm with increasing time.

Bilirubin catabolism in vivo

In plasma, bilirubin is protein bound, specifically to albumin. This albumin-bound bilirubin is known as conjugated while unbound one as unconjugated. Small amount of bilirubin is also conjugated with glucose (Blanckaert *et al.*, 1974) and xylose (Compernolle *et al.*, 1971; Fevery *et al.*, 1977). In the liver, albumin-bound bilirubin is removed from the albumin and taken up at the sinusoidal surface of the hepatocytes by a carrier-mediated saturable system. The smooth endoplasmic reticulum provides a specific set of enzymes which convert bilirubin into diglucuronide. This conversion can also take place in the kidney and the intestinal mucosa. Secretion of this conjugated bilirubin into the bile occurs against a large concentration gradient and is carried out by an active transport mechanism. As the conjugated bilirubin

reaches the terminal ileum and the large intestine, the glucuronides are removed by specific bacterial enzymes and the pigment is subsequently reduced by the fecal flora to a group of colourless tetrapyrrolic compounds, called urobilinogens formed in the colon, oxidized to urobilins and finally excreted in the feces (Schmid & McDonough, 1978).

Bilirubin binding to serum albumin

Serum albumin, a nonglycoprotein is known to bind a variety of substances both cationic and anionic differing in structure and chemical properties (Peters, 1970; 1975; 1980; 1985) and large number of drugs (Brodersen, 1974; Brodersen *et al.*, 1977; Robertson *et al.,* 1991; Onks *et al.*, 1991; Evans *et al.*, 1992). Among the anionic ligands known to bind albumin, long chain fatty acids (Brown & Shockley, 1982), bilirubin (Brodersen, 1979; Tayyab & Qasim, 1988) and tryptophan (Fehske *et al.*, 1979) are physiologically important.

Binding of bilirubin to serum albumin was first reported in 1949 (Martin, 1949). Earlier, it was suggested that one albumin molecule could bind two to three molecules of bilirubin (Martin, 1949; Odell, 1959; Watson, 1962; Schmid *et al.*, 1965). However, others (Ostrow & Schmid, 1963; Kaufmann *et al.*, 1969; Kucerova & Jirsa, 1969; Brodersen, 1979; Jacobsen & Brodersen, 1983) have described that albumin probably has two binding sites for bilirubin, one of which has a much higher affinity than the other. Jacobsen in 1969, using the peroxidase method, prepared the first binding isotherm for bilirubin-

TABLE - I

Binding parameters for bilirubin - HSA interaction

Method	Conditions			n_1	K_1 M^{-1}	n_2	K_2 M^{-1}	Reference
	pH	Ionic strength (M)	Temp.					
Peroxidase	7.4	0.1	37°C	1	$1.4x10^8$	2	$5x10^5$	Jacobsen, 1969
Equilibrium dialysis	-	-	-	-	$2.4x10^7$	-	-	Krasner *et al.*, 1973
CD spectra	8.5	0.50	24°C	1	$6.7x10^6$	1	$3x10^5$	Beaven *et al.*, 1973
Fluorescence quenching	7.4	0.18	25°C	1	$7.0x10^7$	1	$6x10^6$	Chen, 1973
Peroxidase	7.4	0.15	37°C	1	$3.2x10^7$	-	-	Nelson *et al.*, 1974
Peroxidase	7.4	0.15	37°C	1	$(3.8\pm1.5)x10^7$	-	-	Reed *et al.*, 1975
BSA-agarose	7.4	0.15	37°C	1	$(4.2\pm0.3)x10^7$	-	-	Reed *et al.*, 1975
Sephadex	-	-	-	1	$7x10^7$	1	$5x10^{-5}$	Yamaji, 1975
Peroxidase	7.4	0.18	37°C	1	$3x10^7$	-	-	Jacobsen & Brodersen, 1976

Contd...

Method	Conditions pH	Ionic strength (M)	Temp.	n_1	K_1 M^{-1}	n_2	K_2 M^{-1}	Reference
Fluorescence quenching	7.30-7.50	0.35	Amb.room	1	$1.0x10^8$	1	$3x10^6$	Levine, 1977
Peroxidase	7.40	0.18	37°C	1	$5.6x10^7$	-	-	Jacobsen, 1977
Peroxidase	7.40	0.10	37°C	-	$7x10^7$	-	-	Brodersen & Funding, 1977
Spectrophotometry	7.40	0.10	37°C	-	-	2	$5x10^5$	Brodersen & Funding, 1977
Kinetics	7.40	0.10	4°C	1	$>4x10^9$	-	-	Gray & Stroupe, 1978
Fluorescence quenching	7.45	0.07	37°C	1	$6.4x10^7$	1	$2.5x10^6$	Berde *et al.*, 1979
Fluorescence quenching	7.40	0.10	18°C	1	$2.07x10^8$	-	-	Rubaltelli & Jori, 1979
Fluorescence of bilirubin	7.40	0.15	25°C	1	$(7\pm2)x10^6$	-	-	Lamola *et al.*, 1979
	7.40	0.15	37°C	1	$4x10^6$	-	-	Lamola *et al.*, 1979
Fluorescence quenching	7.40	0.15	25°C	1	$(5\pm2)10^6$	-	-	Lamola *et al.*, 1979
Peroxidase	7.40	0.18	37°C	1	$5.5x10^7$	1	$4.4x10^6$	Brodersen, 1979
Spectrophotometry	8.90	0.10	37°C	1	abt $1x10^8$	-	-	Brodersen, 1980

albumin and showed that one molecule of bilirubin is bound with high affinity (Ka= 1.4 x 10^8 litres/mole) and that the isotherm further corresponds to binding of two additional molecules of the pigment with lower affinity (Ka= 5 x 10^5 litres/mole) (Jacobsen, 1969). The binding affinity of bilirubin to the high affinity binding site of albumin is well worked out. Different methods used for the determination of binding constants of the high as well as low affinity binding sites give fairly constant results (see Table I). However, the binding of third molecule of bilirubin has not been demonstrated by most of the techniques.

If has been shown that albumin binds bilirubin reversibly in plasma and even at bilirubin/albumin molar ratio of 1:1, a very small fraction of the total bilirubin remains as unbound fraction (Brodersen, 1979; Wennberg *et al.*, 1979; Wennberg, 1988). The binding affinity of bilirubin to serum albumin has been reported to be independent of pH from 7.0 to 9.0 (Wennberg, 1971; Nelson *et al.*, 1974; Jacobsen & Brodersen, 1976; Wennberg & Rasmussen, 1978; Brodersen, 1979) However, albumin does not bind bilirubin above pH 12.0 because of unfolding of albumin and due to the insolubility of bilirubin at low pH, it is not possible to investigate whether it can be bound to serum albumin or not (Brodersen, 1979). Further, the affinity of bilirubin binding to albumin for primary binding site has been found to decrease with increasing temperature and ionic strength (Jacobsen, 1977). This suggests the role of electrostatic interactions in the binding process.

Bilirubin toxicity and bilirubin encephalopathy

Hervieux in 1847 first described yellow staining of the basal ganglia in association with neonatal jaundice (Hervieux, 1847) and the phenomenon was termed as kernicterus by Schmorl in 1904 (Schmorl, 1904). This term has since been used to describe both the acute, often fatal condition in the newborn with substantial elevation of serum bilirubin levels, seizures, opisthotonus, and bleeding tendency, as well as the neurologic sequelae in survivors, consisting of choreoathetosis, asymmetric spasticity, paresis of upward gaze and neurogenic hearing loss (Byers *et al.*, 1955; Perlstein, 1960; Hyman *et al.*, 1969; Keaster *et al.*, 1969; Fenwick, 1975). But now-a-days, the term kernicterus is replaced by another term bilirubin encephalopathy, so as to include all conditions in which bilirubin is known or thought to be the cause of brain toxicity (Hansen & Bratlid, 1986). Several studies have shown that bilirubin influences several enzymic system (Karp, 1979). It inhibits the binding of cAMP to protein kinase (Constantopoulos & Matsaniotis, 1976). However, rat liver ATP-ase has been reported to be stimulated by bilirubin (Zetterstrom & Ernster, 1956). Glycogenesis is stimulated in the brain of congenitally hyperbilirubinemic rats (Gunn rats) (Karp, 1979). It causes complete inhibition of DNA synthesis in Morris hepatoma cells at concentrations of 170 μM after 10 minutes of exposure (Thaler, 1971). In cultured human fibroblasts, bilirubin combined with phototherapy induced strand breakage in DNA (Rosenstein *et al.*, 1983; Rosenstein & Ducore, 1984).

Nature of bilirubin involved in bilirubin cell toxicity is still a controversy. It has been suggested that kernicterus and bilirubin toxicity are caused by the bilirubin-albumin complex crossing a disrupted blood-brain barrier and that the level of free bilirubin or nature of bilirubin binding may not be important in the development of bilirubin encephalopathy (Levine *et al.*, 1982) . On the other hand, studies on the effects of bilirubin on respiration and viability in cell cultures have shown that toxicity increases when bilirubin is present in the incubate in molar concentrations which exceed that of albumin (Lie & Bratlid, 1970; Bratlid & Rugstad, 1972). Further, if albumin is present in an equimolar or higher ratio with bilirubin, toxicity is blocked (Rasmussen & Wennberg, 1972). Now, it is well recognized that free unconjugated bilirubin is the potential cytotoxic substance and its damaging effect on the cells of the central nervous system in newborns, suffering from a severe form of hyperbilirubinemia (kernicterus) is well known (Metze, 1977; Odell, 1980). Even other cell types and tissues, e.g., platelets, heart, kidney, Langerhans islets of the pancreas, liver and teeth are reported to be damaged by free unconjugated bilirubin (Metze, 1977). Unconjugated bilirubin also induces platelet aggregation (Moiny *et al.*, 1990) and posseses a direct cytotoxic effect for human lymphocytes and granulocytes (Miler *et al.*, 1985). During the *in vitro* interaction between bilirubin and cells, bilirubin undergoes degradation due to both adsorption to the cells and action of cell enzymes (Knobloch & Miler, 1987).

Binding of bilirubin to erythrocytes

Binding of bilirubin to erythrocytes was first reported by Watson (1962) who also demonstrated that presence of certain drugs such as sulphonamide and sodium salicylate greatly enhanced this binding. Later on, several other workers have also studied the binding of bilirubin to erythrocytes and erythrocyte ghosts in greater detail (Oski & Naiman, 1967; Cheung *et al.*, 1966; Kaufmann *et al.*, 1967; Bratlid, 1972 a & b; Barnhart & Clarenburg, 1973; Kapool, 1975; Thaler & Wennberg, 1977; Bouillerot *et al.*, 1981; Sato & Kashiwamata, 1983; Sato *et al.*, 1987; Gulian, 1987; Hayer *et al.*, 1989). The amount of erythrocyte-bound bilirubin is increased either on increasing the bilirubin concentration, while keeping the bilirubin/albumin molar ratio constant or by increasing the bilirubin/albumin molar ratio while keeping the bilirubin concentration constant (Bratlid, 1972 a). At any constant bilirubin/ albumin molar ratio below 1:1, increase in bilirubin concentration leads to a smaller increase in erythrocyte-bound bilirubin. On the other hand, erythrocyte-bound bilirubin is increased around four fold on increasing bilirubin concentration at any bilirubin/albumin molar ratio above 1:1 (Kaufmann *et al.*, 1967; Bratlid, 1972 a; Barnhart & Clarenburg, 1973). Bilirubin binding to erythrocytes takes place within 10 minutes and further increase in the incubation time does not result in any increase in erythrocyte-bound bilirubin (Barnhart & Clarenburg, 1973; Sato & Kashiwamata, 1983). At a given albumin concentration, erythrocytes and albumin bind constant proportions of bilirubin, despite varying bilirubin concentrations (Barnhart & Clarenburg,

1973). However, Wennberg & Rasmussen (1978) have reported that at equilibrium, the cellular content of bilirubin is a function of the concentration of free bilirubin acid and not the total bilirubin concentration or bilirubin/ albumin molar ratio.

Effect of pH on the binding of bilirubin to erythrocytes, erythrocyte ghost and other cell types has been studied earlier by several workers and an increase in erythrocyte-bound bilirubin has been reported with decreasing pH (Bratlid, 1972 b; Nelson *et al.*, 1974; Sato & Kashiwamata, 1983; Wennberg, 1988). In presence of albumin, decrease in pH from 7.4 to 6.8 results in an increase in erythrocyte-bound bilirubin at all the bilirubin/albumin molar ratios, including molar ratios below 1:1 (Bratlid, 1972 b). However, Sato & Kashiwamata (1983) have reported that the saturable binding of bilirubin to the erythrocyte membranes has a pH optimum around 7.1. They have suggested that the cellular susceptibility to bilirubin at lower pH may be determined not only by the physical state of bilirubin but also by the physico-chemical conditions of bilirubin binding sites on the membranes. This has been supported by an earlier finding (Bratlid, 1972 b) that in absence of albumin, at higher bilirubin concentrations, there is an increase in erythrocyte-bound bilirubin on decreasing pH, although at lower bilirubin concentrations, decrease in pH does not affect the erythrocyte-bound bilirubin. Thus, it appears that bilirubin binding to erythrocytes not only depends upon pH but also on bilirubin concentration. Most of the workers (Odell, 1970; Zamet & Chunga, 1971; Bratlid, 1972 b; Maisels, 1972) are of the view that increased cellular binding of bilirubin at

lower pH may be due to either increased cellular affinity for bilirubin or increased dissociation of bilirubin from the secondary binding sites of albumin at low pH without any reduction in the affinity of the primary binding site.

The molecular basis of bilirubin binding mechanism to erythrocytes is still not clear. But, there are some clues which are important for elucidation of this mechanism. It is reported that bilirubin is not solely bound to the membranes but that its greater part is also present in the cytosol of erythrocytes (Lamola *et al.*, 1979). This is also supported by the finding that stroma binds less bilirubin as compared to the whole cells, which means that some of the bilirubin might bound to other structures other than the stroma (Bratlid, 1972 a). Albumin-bound bilirubin could not penetrate into the cell membranes whereas unconjugated bilirubin can diffuse freely through biological membranes as it has been found accumulated in the intracellular compartments such as mitochondria (Odell, 1965, 1966; 1970; Wennberg, 1988). The same amount of bound bilirubin is reported in both red blood cells and erythrocyte ghosts having same phospholipid content (Hayer *et. al.* 1989). Thus, it appears that unlike other cells, erythrocytes have all the bilirubin as membrane-bound. Bilirubin binds to the specific binding sites present in the membrane. They have reported the binding saturation of 110×10^{-18} moles of bilirubin per red blood cell and the dissociation constant of bilirubin/receptor complex as 170×10^{-6} mol/l (Hayer *et al.*, 1989). Sato & Kashiwamata (1983) have reported the apparent dissociation constant (Kd) and maximum binding (B_{max}) values for the saturable binding of bilirubin as 2.3 μM and 0.93 nmol/mg of membrane

protein respectively. Individual membrane components responsible for the binding of bilirubin have not been characterised yet. However, no difference has been found in between erythrocytes from patients with glucose-6-phosphate dehydrogenase deficiency and erythrocytes from normal hyperbilirubinemic infants, with respect to bilirubin binding (Schettini & Meloni, 1964). Similarly, no difference has been reported in bilirubin binding capacity of erythrocytes from newborn infants with erythroblastosis from that of normal erythrocytes at bilirubin/albumin molar ratios below 1:1. But above molar ratios 1:1, the patient erythrocytes bind little less bilirubin (Bratlid, 1972 a). The bilirubin binding sites in erythrocyte membranes appear not to be composed of proteins as the binding capacity increased after tryptic digestion and heat treatment of these membranes (Sato & Kashiwamata, 1983). Further, affinity labelling of the membrane proteins by bilirubin has not been successful in identifying any specific bilirubin binding protein (Sato & Kashiwamata, 1983). They have suggested that membrane proteins may function as an effective barrier to the binding of bilirubin. Nagaoka & Cowger (1978) have suggested that bilirubin is bound to the lipid bilayers through strong ionic interactions between a cationic head group of the lipid and anionic bilirubin. Binding constant has been reported highest for sphingomyelin as 3.7 $x10^6M^{-1}$, and for diphosphatidylcholine with 5% cholestrol as 2.6 x 10^6 M^{-1}. Bilirubin-phosphatidylcholine complex formation as reported by Mustafa & King (1970), is also reported to be formed in diethyl ether solution (Brodersen, 1979). Sato *et al.* (1987) have reported that neuraminidase and phospholipase D digestions

do not effect the binding capacities, but phospholipase C treatment greatly enhances the bilirubin binding. Further, inside-out sealed erythrocyte vesicles show comparable binding capacities with the right-side-out sealed membranes. On the basis of their results, these authors have rejected the idea of the involvement of polar headgroups of phospholipids as the binding sites for bilirubin on the plasma membranes as suggested by Mustafa & King (1970) and Nagaoka & Cowger (1978). They have suggested that the negatively charged phosphoric acid moiety of phospholipids prevents a large amount of bilirubin from binding to the membranes and that the bilirubin binding sites might be distributed on both outer and inner surface of the membranes, or might exist in the membranes where bilirubin might be accessible from either side.

From the above review of the literature, it is clear that erythrocytes have saturable binding sites for bilirubin. Despite extensive studies, the nature of these receptors has not been defined. Further, there is no report on whether erythrocytes from different mammalian species bind same amount of bilirubin or not. Since there is a difference in protein and lipid make-up of erythrocyte membranes of different mammalian species (Lenard, 1970; Barenholz & Thompson, 1980), it would be interesting to carry out detailed studies on the binding of bilirubin to erythrocytes of different mammalian species. This will be helpful in characterizing the receptors for bilirubin present on these cells. With this idea in mind, I decided to undertake a detailed study on the binding of bilirubin to erythrocytes from different mammalian species, namely, human,

buffalo, goat and sheep both in the absence and in the presence of their serum albumins respectively. This dissertation describes a report on the comparative studies on bilirubin binding to erythrocytes from different mammalian species under four different conditions i.e. (i) at different bilirubin/albumin molar ratio (keeping bilirubin concentration constant while varying the albumin concentration) (ii) at different bilirubin/albumin molar ratios (keeping albumin concentration constant while varying bilirubin concentration) (iii) at increasing bilirubin concentrations (keeping bilirubin/ albumin molar ratio constant while varying both bilirubin and albumin concentrations and (iv) in absence of albumin.

The results of this study suggest that goat erythrocytes bind highest amount of bilirubin followed by buffalo and human while sheep erythrocytes bind lowest amount of bilirubin.

Future plan of work

Although, the amount of bilirubin bound to erythrocytes from different mammalian species is different as shown in this study , but the molecular organization of binding sites in erythrocyte membranes, such as the involvement of proteins, lipids, carbohydrates as well as the effect of erythrocyte volume and intracellular Ca^{++} content, are poorly understood. Therefore, in future, I would like to study in detail the binding of bilirubin to erythrocytes of human, goat, buffalo and sheep under the following conditions:

(i) Bilirubin binding studies with different age groups of red blood

cells.

(ii) Bilirubin binding studies with trypsinized red blood cells, phospholipase-treated red cells and sialidase-treated red blood cells.

(iii) Bilirubin binding studies in presence of anion transport inhibitor.

(iv) Bilirubin binding studies with $CaCl_2$-treated red blood cells.

EXPERIMENTAL

Materials

Proteins

Bovine serum albumin fraction V, (Lot No. 100 F - 0249) was purchased from Sigma Chemical Company, USA. Human, buffalo, sheep and goat plasma albumins were isolated by the method of Tayyab and Qasim (1990).

Reagents used in protein determination

Sodium carbonate, sodium potassium tartarate, lithium sulphate and liquid bromine (all GPR grade) were obtained from B.D.H., India. Copper sulphate, sodium tungstate and sodium molybdate (all LR grade) were purchased from s.d. fine chem., India. Qualigens Fine Chemicals, India was the source of orthophosphoric acid and hydrochloric acid.

Reagents used in bilirubin determination

Reagents used in bilirubin determination with their sources in parentheses were: Caffeine anhydrous (LR), sulphanilic acid (LR), sodium nitrite (AR) (s.d. fine chem., India); sodium acetate (AR) (Qualigens Fine Chemicals, India); sodium hydroxide (GPR), sodium potassium tartarate (GPR) (B.D.H., India) and sodium benzoate (GR) (Loba Chemie, India).

Reagents used in polyacrylamide gel electrophoresis

Acetic acid glacial (AR), amido black 10B (LR), glycerol (LR), charcoal (GR) and methanol (GR) were purchased from s.d. fine chem., India.

Acrylamide (LR), N, N'-methylenebisacrylamide (LR) and N, N, N', N'-tetramethylethylenediamine (LR) were obtained from S.R.L., India. B.D.H., India was the source of ammonium persulphate (GR), tris (hydroxymethyl) aminomethane (LR) and glycine (LR). Bromophenol blue was obtained from B.D.H. England.

Miscellaneous

Various chemicals/reagents used in this study with their sources in parentheses were : ammonium sulphate (LR), ammonia solution, sodium chloride (AR) (Qualigens Fine Chemicals, India); bilirubin (GR), potassium dichromate (AR), potassium hydrogen phthalate (LR), sodium azide (LR), sodium phosphate (mono and dibasic) (LR) (s.d. fine chem., India) and sodium carbonate (LR), tri-sodium citrate (LR), sulphuric acid (LR) (B.D.H., India).

Dialysis membranes of 1 inch diameter were obtained from Sigma Chemical Company, USA and Whatman filter paper No. 1 was purchased from Whatman Lab. Div., Kent, England.

All glass distilled water was used throughout these studies. All the experiments were perfomed at 25°C unless otherwise stated.

AR = Analytical Reagent; GR = Guaranteed Reagent;
LR = Laboratory Reagent; GPR = General Purpose Reagent.

Methods

pH measurements

pH measurements were made on a digital pH meter, type DPH-100 using DPH-100 combined electrode. The pH meter was routinely calibrated with 0.05 M potassium hydrogen phthalate buffer, pH 4.0 at 25°C in the acidic range and with 0.01 M sodium tetraborate buffer, pH 8.2 at 25°C in the alkaline range.

Optical measurements

Light absorption measurements were made on either Cecil single beam spectrophotometer, model CE-202 or Photochem-8 colorimeter using quartz or glass cuvettes of 1 cm path length.

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. This method involves the use of two reagents, namely, Folin & Ciocalteu's phenol reagent and copper reagent. These reagents were prepared in the following manner.

(i) ***Preparation of copper reagent*** : The following stock solutions were prepared.

(1) 4% (w/v) sodium carbonate

(2) 4% (w/v) sodium potassium tartarate

(3) 2% (w/v) copper sulphate.

In order to prepare the copper reagent, the solutions (1), (2) and (3) were mixed in the ratio of 100 : 1 : 1 (v/v/v). First the solution (2) was added to solution (1) and after mixing, solution (3) was added to the mixture of (1) and (2). The sequence of mixing the solutions was essential to avoid precipitation. The reagent was filtered before use.

(ii) ***Preparation of Folin & Ciocalteu's phenol reagent*** : The reagent was prepared by the method of Folin and Ciocalteu (1927). One hundred grams of sodium tungstate, 25 gm of sodium molybdate, 50 ml of 85%(v/v) orthophosphoric acid, 100 ml of 11.3 N hydrochloric acid and 700 ml of water were mixed together and the contents were refluxed gently in a two litre round bottom flask wrapped with black paper for ten hours. After cooling, 150 gm lithium sulphate, 50 ml of water and few drops of liquid bromine were added. The mixture was boiled without condenser for 30 minutes to remove excess bromine. The solution was cooled, filtered and diluted to one litre. The yellow coloured reagent was stored in an amber coloured bottle. The stock solution was diluted five times with water before use.

(iii) ***Procedure*** : An appropriate volume of standard protein solution (4 mg/ 10 ml) in the range of 0.1 -1.0 ml was taken in a series of test tubes and the total volume was made upto 1.0 ml with water or buffer. Then 5.0 ml of freshly prepared copper reagent was added to all the tubes and after thorough mixing, these were left at room temperature for 10 minutes. Then 1.0 ml of diluted Folin & Ciocalteu's phenol reagent was

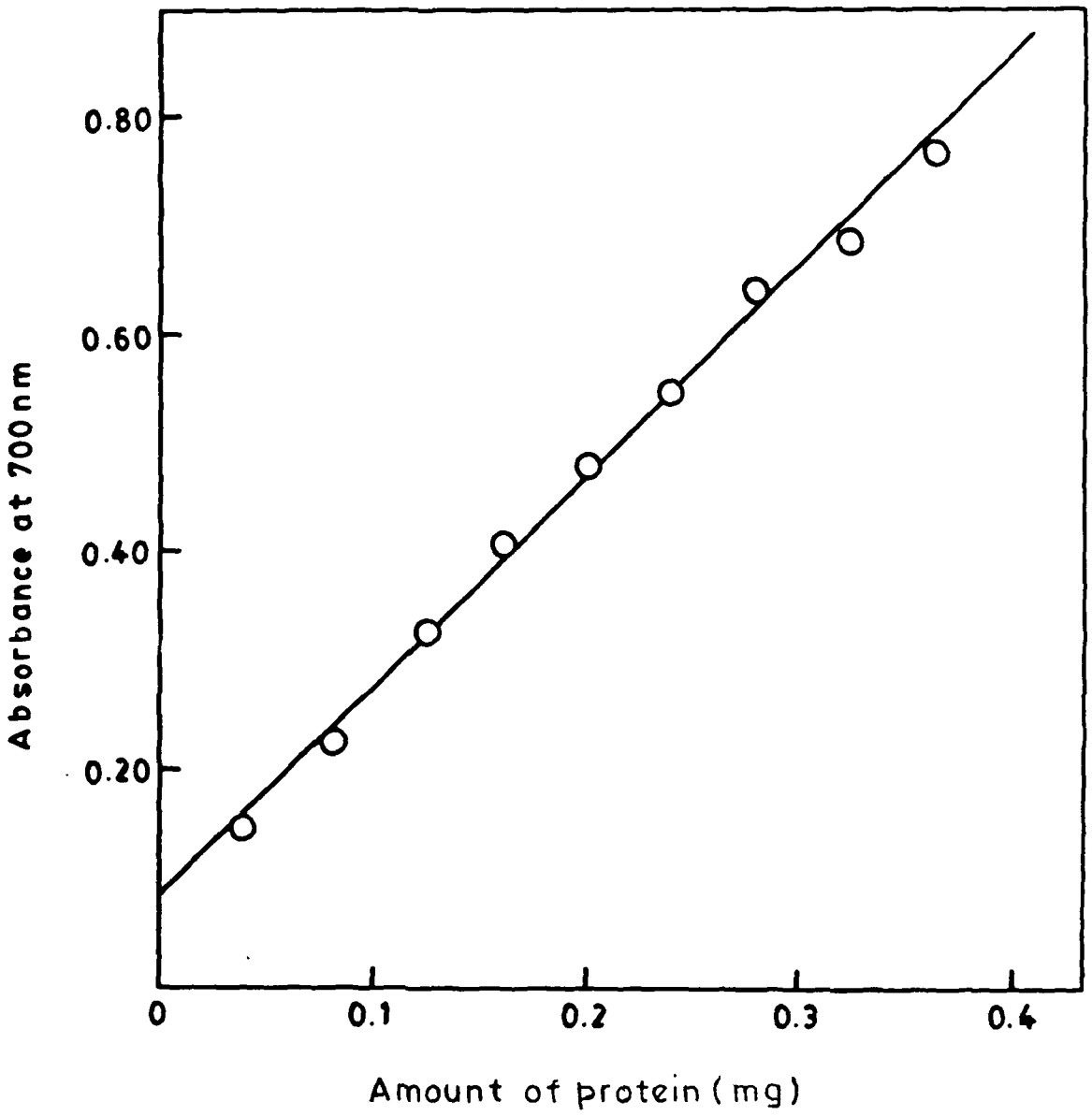

Figure 2. Standard curve for the determination of protein concentration by the method of Lowry *et al.* (1951).

added and the contents were mixed well. After 30 minutes, the colour intensity was read at 700 nm against a blank prepared in the same way except that instead of protein solution, 1.0 ml of water or buffer was taken. A graph was plotted between absorbance at 700 nm and amount of protein (Fig. 2), which yielded the following straight line equation :

$(O.D)_{700\ nm}$ = 1.9 (amount of protein, mg) + 0.08

Isolation of plasma albumin

Plasma albumin was isolated by the method of Tayyab and Qasim (1990). Heparinized human blood was obtained from the Blood Bank of Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh and citrated blood of goat, buffalo and sheep were collected from the slaughter house. Blood was centrifuged at 3000 rpm for 20 minutes to obtain plasma. To each 500 ml of plasma in a one litre conical flask was added sufficient amount of 4 M ammonium sulphate solution (whose pH had been previously adjusted to 7.0 with ammonium hydroxide) to make the plasma 2.26 M in ammonium sulphate. Each mixture was kept for 12 hours at room temperature and then centifuged at 6000 rpm for 30 minutes. The precipitate was discarded and the supernatant thus obtained was carefully diluted with water such that the final concentration of ammonium sulphate was reduced to 1.9 M and the pH was adjusted to 4.1 with 0.5 N sulphuric acid. After incubation at room temperature for about 12 hours, the precipitate was collected by centrifugation at 6000 rpm for 30 minutes. It was washed three times with 2.2 M ammonium

sulphate sulution, pH 4.2 and then dissolved in 0.07 M sodium phosphate buffer containing 0.08 sodium chloride, pH 7.4. The protein, thus obtained, was extensively dialysed against 6 litres of 0.07 M sodium phosphate buffer containing 0.08 M sodium chloride, pH 7.4. The preparation was stored at 4°C and concentration was determined by the method of Lowry *et al.* (1951).

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in tris- glycine buffer, pH 8.2, ionic strength 0.02 according to the method of Davis (1964). The gel tubes (0.5 x 7.5 cm) were washed with detergent, chromic acid and finally with water. The dried tubes were siliconized with a solution of 5% (v/v) dimethyl dichlorosilane in chloroform. Then the tubes were fixed vertically in a stand by keeping the lower ends closed with the help of stoppers. A small pore solution containing 7% (w/v) acrylamide, 0.18% (w/v) N, N'- methylenebisacrylamide, 0.03% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.07% (w/v) ammonium persulphate was prepared. To each tube, 1.0 ml of the above solution was poured. The surface of the small pore solution was carefully layered with few drops of water. After 30 minutes of incubation at room temperature (sufficient for polymerization), water layer was removed using strips of filter paper. The surface was rinsed with small amount of electrophoretic buffer. For sample preparation, 200 μgm protein was taken in about 0.1 ml of electrophoretic buffer containing one drop of glycerol. Gel tubes were fitted in an electrophoresis assembly. About 0.03 ml of the sample was applied and the empty space left in the tube was carefully filled with the

electrophoretic buffer. Few drops of a 0.01% (w/v) bromophenol blue in 0.1 N sodium hydroxide (w/v) solution were added in the upper chamber and then the buffer was added in a volume such that the gel tubes were completely dipped. An anodic current of 2-4 milliampere per tube was passed till the bromophenol blue front had migrated to nearly three fourth of the gel length. After 2 hours, electrophoresis was terminated and the gels were taken out from the gel tubes and stained for one hour with 1% (w/v) amido black solution prepared in 7% (w/v) acetic acid. The position of tracking dye was marked by inserting a small piece of wire before placing these gels in staining solution. Destaining was performed mechanically by shaking the tubes with 7% (v/v) acetic acid at 37°C.

Determination of bilirubin concentration

Bilirubin concentration was determined according to the method of Jendrassik and Grof (1938) as modified by Fog (1958). The method involves the use of three reagents, namely, I, II and III which were prepared in the following manner:

(i) ***Preparation of reagent I*** : Reagent I was prepared by dissolving 20 gm of caffeine anhydrous, 30 gm of sodium benzoate and 50 gm of sodium acetate in 400 ml of water. The mixture was warmed upto 50°C with continuous stirring until a clear transparent solution was obtained.

(ii) ***Preparation of reagent II*** : Reagent II consists of two parts, i.e. reagent IIa and IIb.

(a) ***Reagent IIa*** : Reagent IIa was prepared by dissolving 0.5 gm of sulphanilic acid in 1.5 ml of concentrated hydrochloric acid followed by dilution with 100 ml of water. Heating was avoided to prevent crystal formation.

(b) ***Reagent IIb*** : Reagent IIb was prepared by dissolving 500 mg of sodium nitrite in 100 ml of water.

Working solution of reagent II was prepared fresh just before the experiment by adding 3 drops of reagent IIb in 5 ml of reagent IIa.

(iii) ***Preparation of reagent III*** : It was prepared by dissolving 30 gm of sodium hydroxide and 105 gm of sodium potassium tartarate in 300 ml of water.

Preparation of standard solution of bilirubin : It was prepared by dissolving few crystals of bilirubin in 1 N NaOH containing 5 mM EDTA and diluting it with 0.06 M sodium phosphate buffer, pH 8.0. Bilirubin concentration was determined spectrophotometrically using a molar extinction coefficient of 47,500 at 440 nm (Jacobsen & Wennberg, 1974).

Procedure : In a series of tubes, increasing volumes of stock bilirubin solution (116 μM) ranging from 0.1 to 1.0 ml were added and the final volume was made upto 1.0 ml with water. It was followed by the addition of 2.0 ml of reagent I and 0.5 ml of reagent II. The contents were mixed well and kept at room temperature for 10 minutes. Then, 1.5 ml of reagent III was added

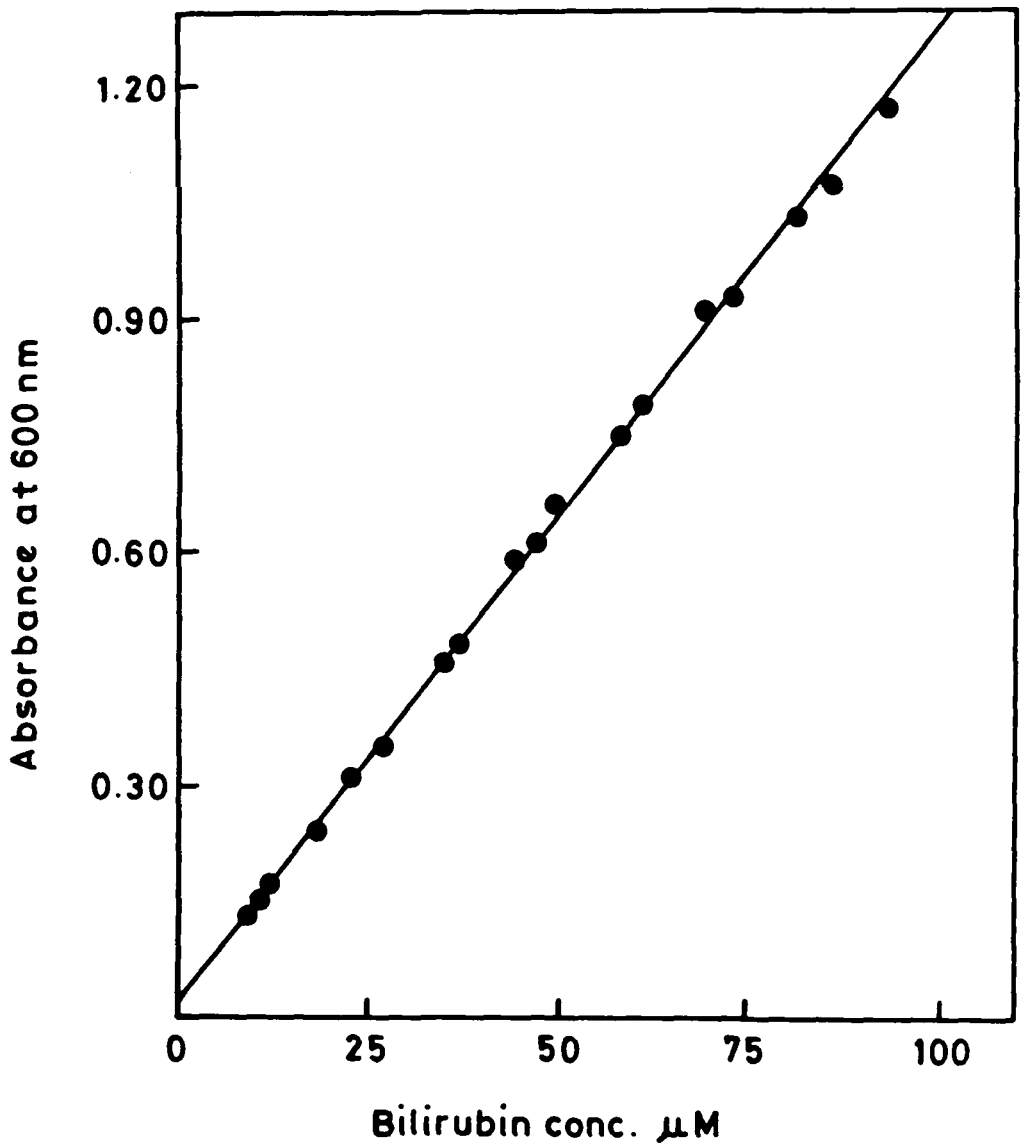

Figure 3. Standard curve for the determination of bilirubin concentration by Fog's method (1958).

in all the tubes and tubes were shaken well. Absorbance of the solution was read at 600 nm. The colour was stable for 30 minutes and incubation of the solution longer than 30 minutes resulted in the appearance of turbidity in all solutions. For each bilirubin concentration, reagent II blank and bilirubin blank were prepared in the same way as described above except that water was used instead of reagent II and bilirubin in these blanks respectively. Absorbance of diazotized bilirubin solution was determined by subtracting the absorbance values of reagent II blank and bilirubin blank from the absorbance of bilirubin solution. Data were plotted as absorbance at 600 nm versus bilirubin concentration, which yielded the following straight line equation (Fig. 3).

$$(O.D.)_{600nm} = 0.0125 \times \text{bilirubin conc. } (\mu M) + 0.02$$

In order to check the interference of plasma albumin in the determination of bilirubin concentration by Fog's method (1958), the bilirubin solution (770 μM) was diluted 10 times with isolated plasma albumin in 0.07 M sodium phosphate buffer containing 0.08 M sodium chloride, pH 7.4. The diluted bilirubin-albumin solution was used instead of stock bilirubin solution and the assay was performed in the same way as described above for bilirubin alone. The standard curve (Fig. 3), thus obtained, showed no change in the value of slope. Therefore, the standard curve of bilirubin can also be used for determination of bilirubin concentration in presence of albumin.

Similarly, use of stock bilirubin solution prepared in 1 N NaOH containing 5 mM EDTA and diluting it with 38 mM sodium carbonate solution

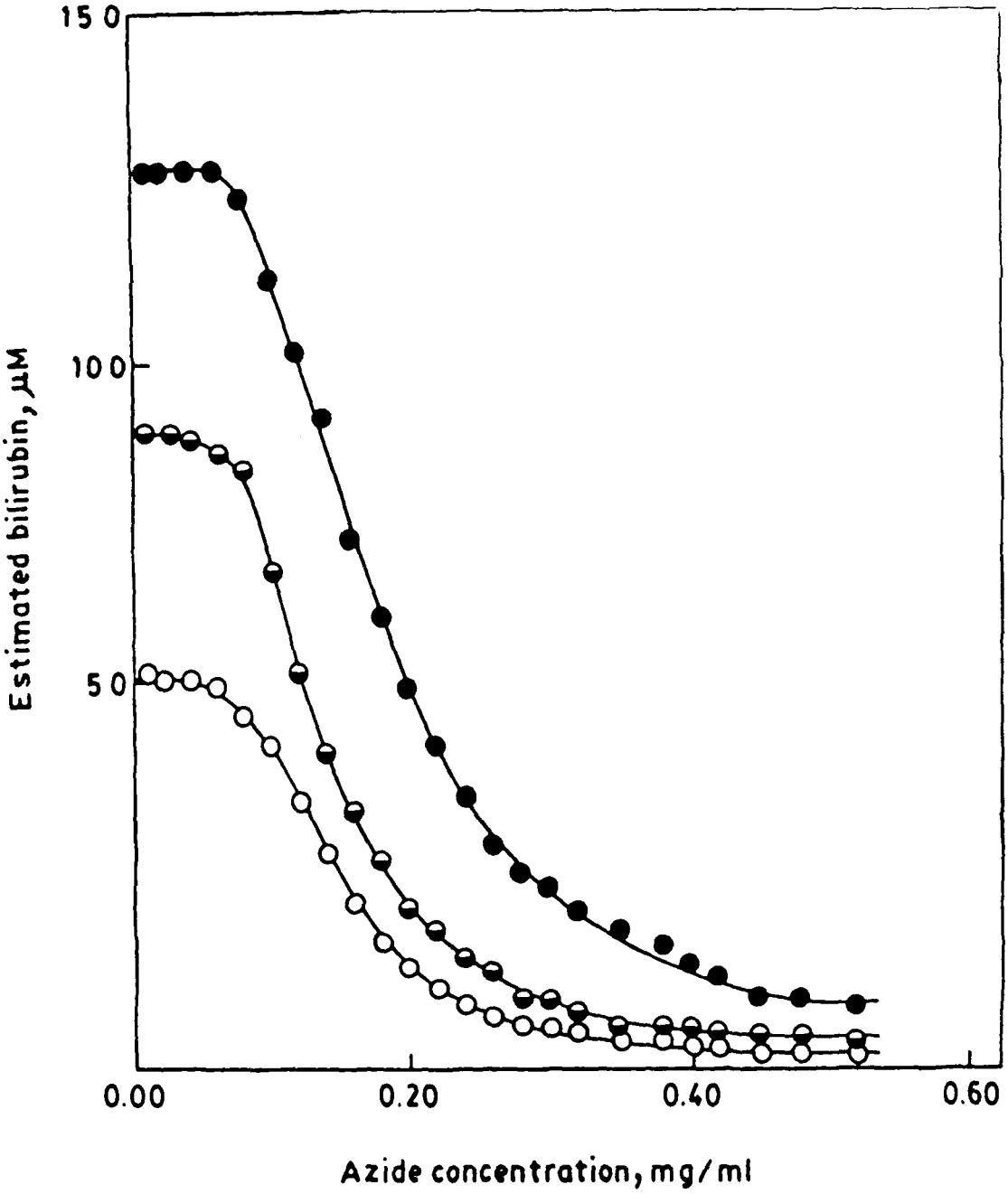

Figure 4. Effect of increasing concentrations of sodium azide on total bilirubin determination by Fog's method (1958). The concentration of bilirubin was 127.0 μM (●), 90.3 μM (◐), and 56.0 μM (○).

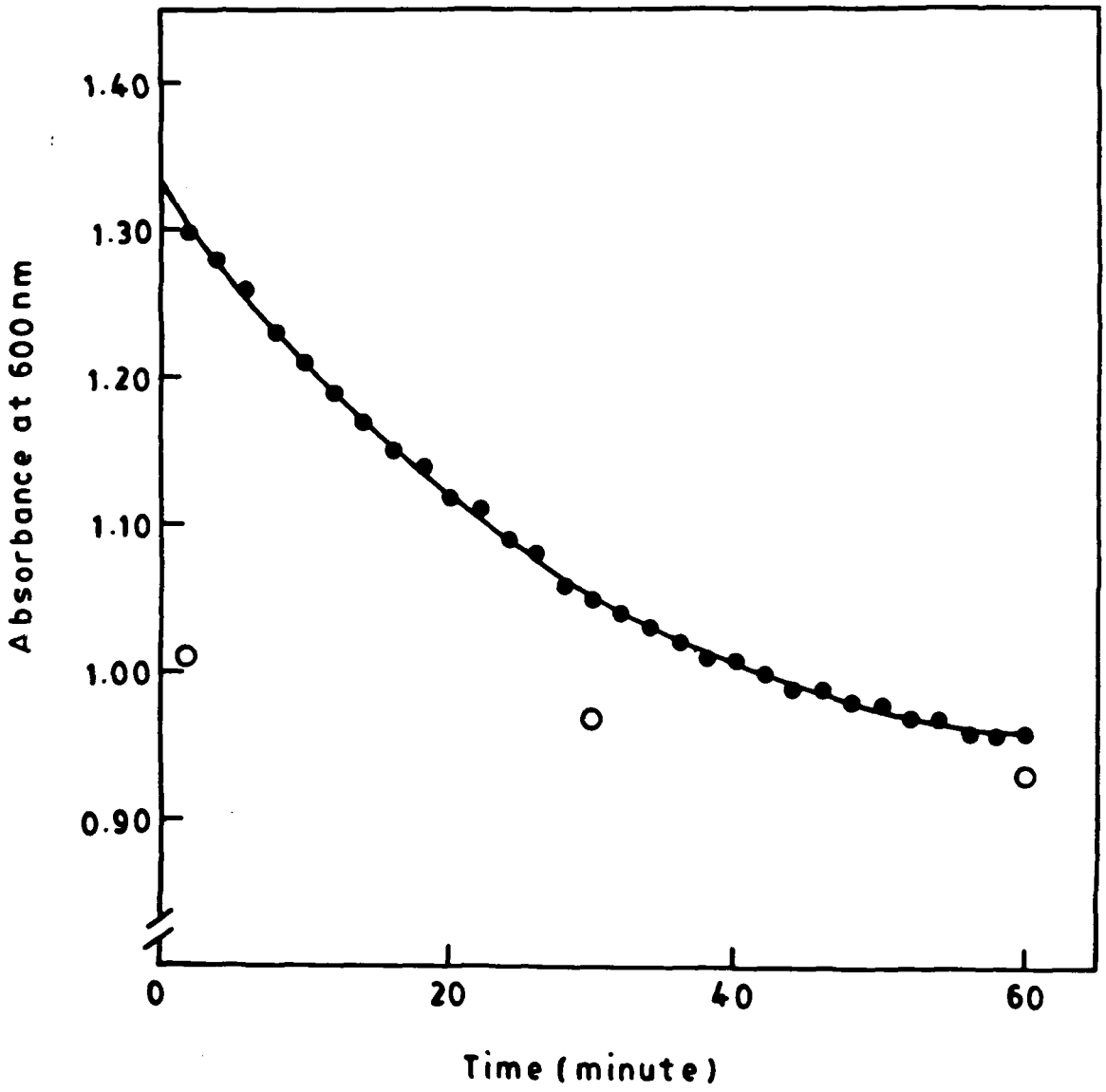

Figure 5. Effect of haemoglobin on the determination of bilirubin concentration by Fog's method (1958) (●) test and (0) azoblank.

containing 5 mM EDTA, pH 11.0 instead of 0.06 M sodium phosphate buffer, pH 8.0 did not show any change in the slope of standard curve using Fog's method (1958).

Effect of sodium azide on the determination of bilirubin concentration by Fog's method

Sodium azide, a bacterio-static agent, used as preservative in many biological samples, was found to be highly interfering substance in the determination of bilirubin by Fog's method (1958). This interference was studied by taking a fixed concentration of bilirubin (127.0, 90.3 or 56.0 μM) and increasing the amount of azide upto 0.50 mg/ml in these samples. A pronounced interference was noted at high azide concentrations. At an azide concentration of 0.50 mg/ml, there was about 96% decrease in estimated bilirubin at all the three bilirubin concentrations used in this study (Fig. 4). Therefore, use of sodium azide was avoided throughout these experiments.

Effect of haemoglobin on the determination of bilirubin concentration by Fog's method

Fog's method (1958) used in this study for determination of bilirubin concentration was also found to be affected by the presence of haemoglobin. In presence of haemoglobin, absorbance of bilirubin solution after Fog's reaction, decreased continuously with time while absorbance of reagent II blank remained constant with time (Fig. 5). However, at varying concentrations of haemoglobin, its value varied. Therefore, use of haemolytic blood samples was avoided in these studies.

Interaction of bilirubin with red blood cells

For studying red cell binding of bilirubin, red blood cells (RBC) with a haematocrit value of 50% were used which were prepared as follows :

(i) ***Preparation of 50% haematocrit value of red blood cells*** : Citrated/heparinized blood, collected from slaughter house/blood bank, was centrifuged in Remi-R 8C centrifuge at 2000 rpm for 20 minutes and the supernatant containing serum was discarded. The white buffy coat above the upper surface of packed RBCs was also removed through gentle decantation. Red blood cells were washed three times with 0.07 M sodium phosphate buffer containing 0.08 M NaCl, pH 7.4. The final packed red blood cells were collected and resuspended in the same buffer at a ratio of 1 : 1 to obtain 50% haematocrit value. The preparation was kept in refrigerator for further use.

(ii) ***Preparation of bilirubin solution*** : Fresh bilirubin solution was prepared by dissolving few crystals in the desired volume of 38 mM sodium carbonate solution containing 5 mM EDTA, pH 11.0. Bilirubin solution was protected from light and experiments with bilirubin were performed in yellow light. The concentration of bilirubin was determined by Fog's method (1958) as described earlier. Bilirubin solution was used within one hour.

(iii) ***Procedure*** : Binding of bilirubin to red blood cells was studied at pH 8.0, ionic strength 0.414 and at 37°C according to the method of Bratlid (1972a). To 1.0 ml of albumin solution in 0.07 M sodium phosphate

buffer containing 0.08 M NaCl, pH 7.4, 1.0 ml of bilirubin solution was added and the volume was made to 5 ml with 0.07 M sodium phosphate buffer containing 0.08 M NaCl, pH 7.4. Then 1.0 ml of red blood cell suspension of 50% haematocrit value was added to it and the tubes were incubated for 30 minutes at 37°C after gentle shaking. After incubation, they were centrifuged at 2000 rpm for 20 minutes and the supernatant was removed by gentle decantation. The red blood cells were washed thrice with 0.07 M sodium phosphate buffer containing 0.08 M NaCl, pH 7.4 such that the last supernatant was devoid of yellow colour. The supernatant was carefully removed and then 2.5 ml of 2.5% albumin solution was added. The erythrocyte-bound bilirubin was eluted by incubation for 30 minutes at 37°C after shaking the tubes gently. The tubes were centrifuged at 2000 rpm for 20 minutes and the supernatant was carefully removed by suction and then subjected to Fog's method for bilirubin determination in the eluate.

In some experiments, the bilirubin concentration was varied [(i) from 6.5 to 38.8 μM (ii) from 13.8 to 78.3 μM and (iii) from 20.0 to 116.7 μM] and albumin concentration kept constant [(i) 13.2 μM (ii) 26.3 μM and (iii) 39.5 μM] to obtain different bilirubin/albumin molar ratios. In other experiments, albumin concentration was varied [(i) from 14 to 84 μM (ii) from 28 to 165 μM and (iii) from 42 to 250 μM] and bilirubin concentration kept constant [(i) 41.8 μM (ii) 83.7 μM and (iii) 125.5 μM] to obtain different bilirubin/albumin molar ratios. In an

experiment, both the bilirubin and albumin concentrations were varied to obtain a constant bilirubin/albumin molar ratio. Experiments without albumin were also conducted. In these experiments, instead of 1.0 ml of albumin solution, 1.0 ml of 0.07 M sodium phosphate buffer containing 0.08 M NaCl, pH 7.4 was taken to keep the final volume of the incubation mixture as 6.0 ml.

RESULTS AND DISCUSSION

Isolation of plasma albumin

Plasma albumins of human, sheep, goat and buffalo were isolated as described in the experimental section. About 2 gm protein was obtained from 100 ml of the plasma. The preparation showed a fair degree of homogeneity as evidenced by the electrophoretic pattern in which the isolated plasma albumins of human, goat, buffalo and sheep gave a single band (Fig. 6).

Binding of bilirubin to erythrocytes

Binding of bilirubin to erythrocytes of different mammalian species was studied under different conditions as described in the experimental section. The results obtained are described below.

(a) At different bilirubin/albumin molar ratios (keeping the bilirubin concentration constant while varying the albumin concentration)

At different bilirubin/albumin molar ratios in the range of 0.5 to 3.0. obtained by taking fixed amount of bilirubin (41.8 μM) and increasing amounts of albumin, the binding of bilirubin to erythrocytes increased continuously with the increase in bilirubin/albumin molar ratio as shown in Fig. 7. Similar pattern of binding was observed with all the four mammalian species studied. At two other fixed bilirubin concentrations (83.7 μM and 125.5 μM), the binding patterns were qualitatively similar

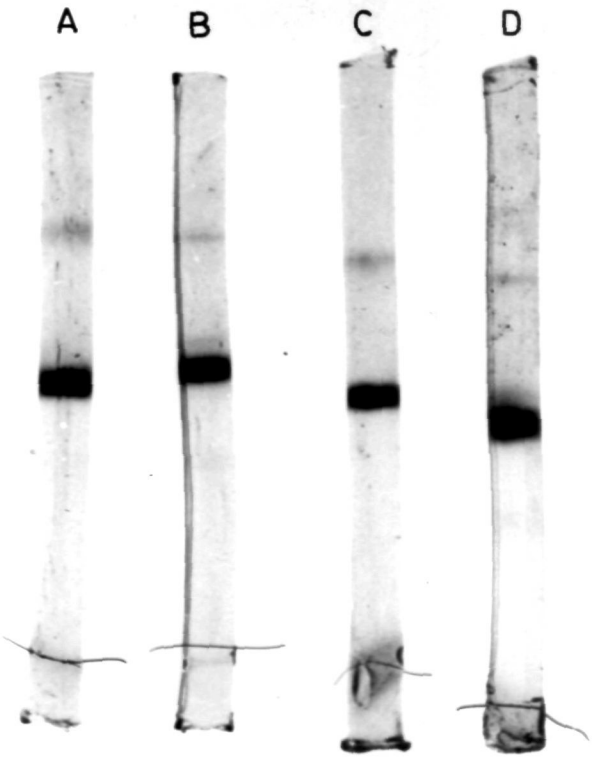

Figure 6. Polyacrylamide gel electrophoresis of different plasma albumins. (A) Goat, (B) Buffalo, (C) Human and, (D) Sheep plasma albumins.

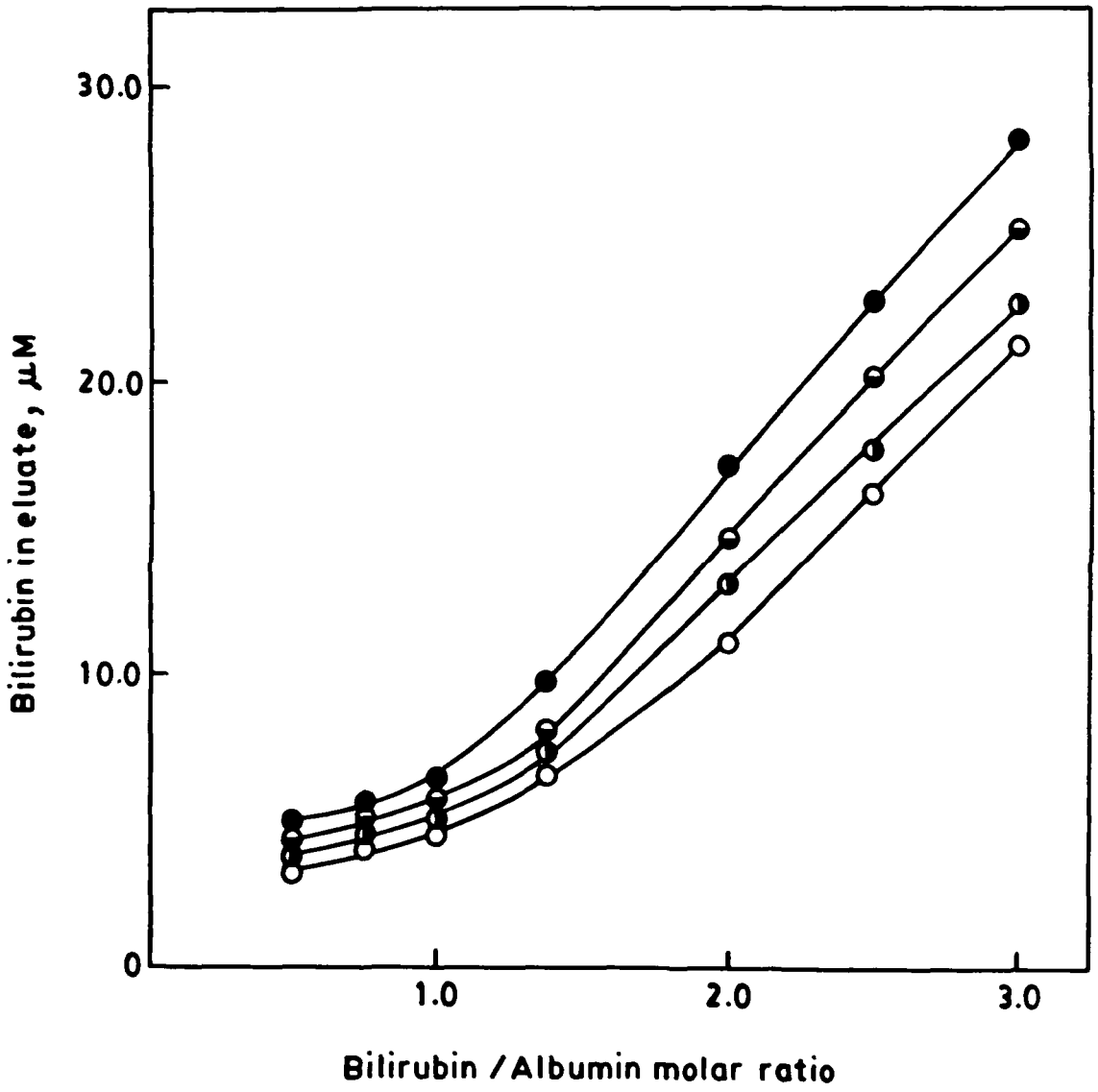

Figure 7. Binding of bilirubin to the erythrocytes of goat (●–●), buffalo (◐–◐), human (◑–◑) and sheep (○–○) at different bilirubin/albumin molar ratios. Bilirubin concentration was kept constant at 41.8 μM and the albumin concentration varied.

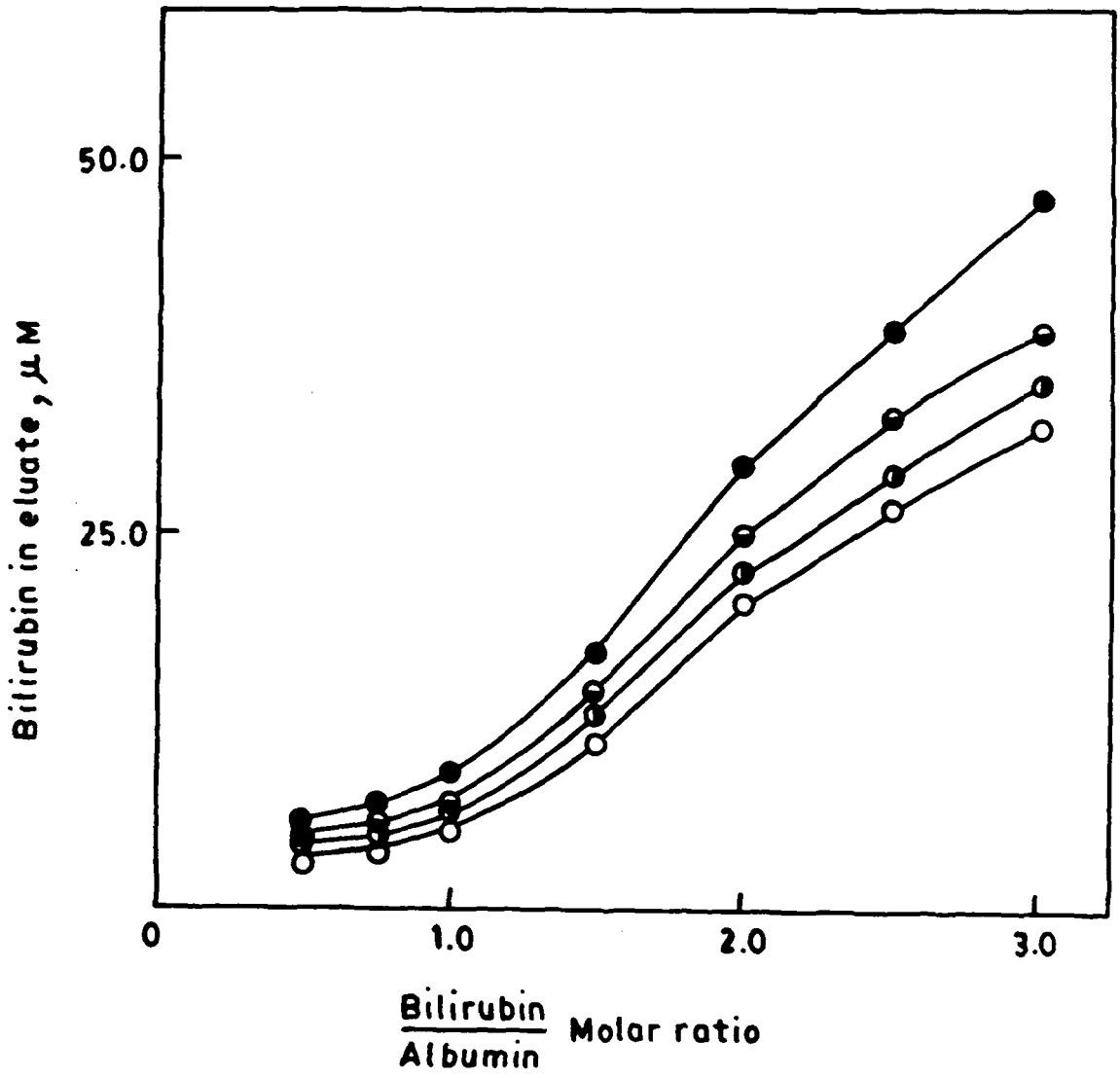

Figure 8. Binding of bilirubin to the erythrocytes of goat (●–●), buffalo (●–●), human (●–●) and sheep (0–0) at different bilirubin/albumin molar ratios. Bilirubin concentration was kept constant at 83.7 μM and the albumin concentration varied.

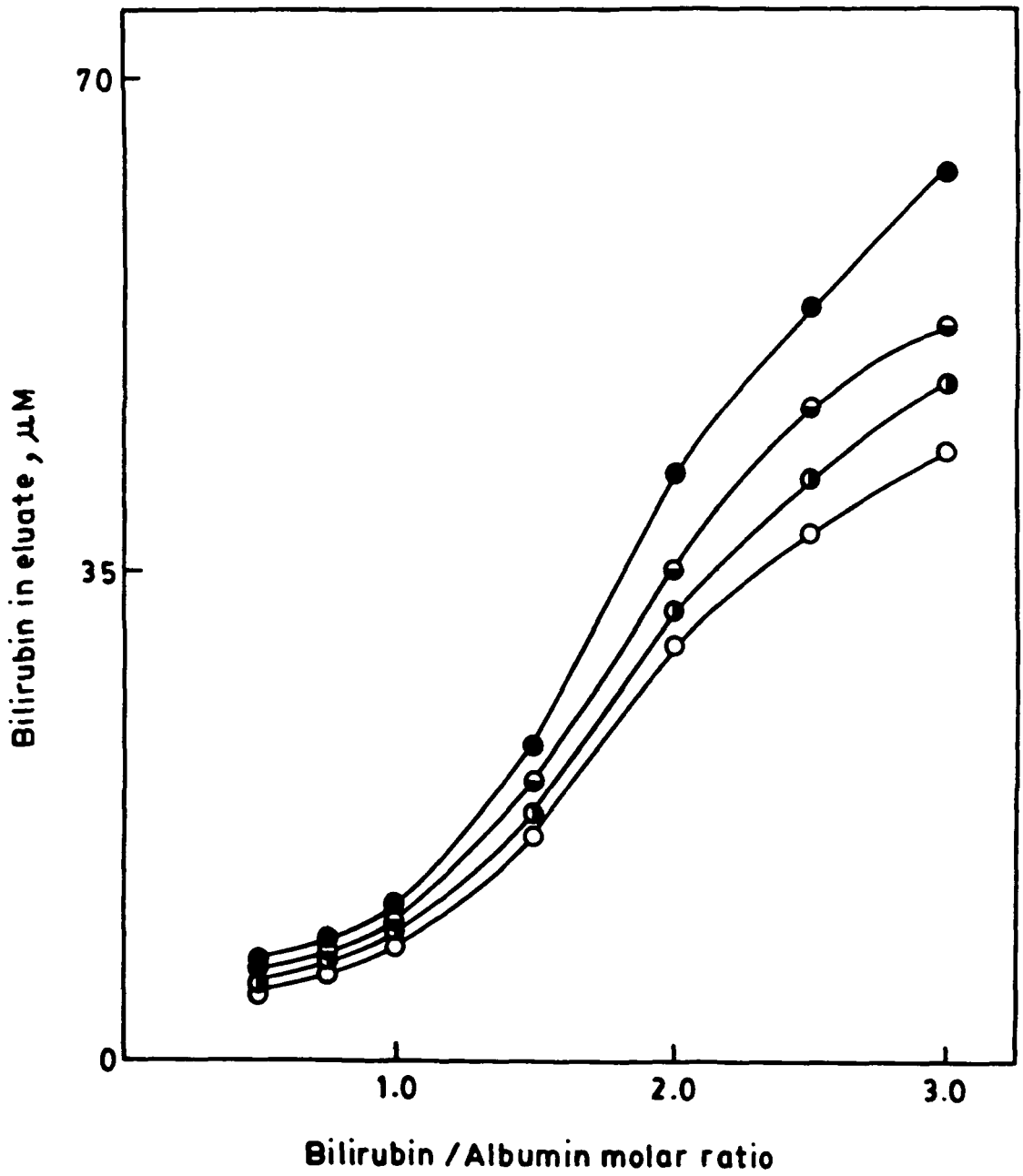

Figure 9. Binding of bilirubin to the erythrocytes of goat (●–●), buffalo (◐–◐), human (◑–◑) and sheep (○–○) at different bilirubin/albumin molar ratios. Bilirubin concentration was kept constant at 125.5 μM and the albumin concentration varied.

to the one obtained with 41.8 μM bilirubin concentration. At any molar ratio, amount of erythrocyte-bound bilirubin increased on increasing bilirubin concentration (see Figs. 7 - 9).

Bilirubin binding patterns as obtained with human erythrocytes were fairly comparable with that of earlier reports obtained with human system (Bratlid, 1972a; Kaufmann *et al.,* 1967; Hayer *et al.,* 1989). However, the amount of erythrocyte-bound bilirubin, in case of human system was smaller than that reported earlier (Bratlid, 1972a). This may be due to the effect of pH on this interaction as we have studied this binding at pH 8.0 while in an earlier study, pH was kept at 7.4 (Bratlid, 1972 a). Several earlier reports suggest a decrease in bilirubin binding by erythrocytes on increasing pH (Bratlid, 1972b; Sato & Kashiwamata, 1983). A comparison of bilirubin binding to erythrocytes obtained from different species suggests significant variation in bilirubin binding at all molar ratios. At a given molar ratio, goat erythrocytes bind maximum amount of bilirubin while sheep erythrocytes bind the lowest amount (see Figs. 7 - 9). The amount of bilirubin bound by buffalo and human erythrocytes was in between the values obtained with goat and sheep erythrocytes. Increase in erythrocyte-bound bilirubin on increasing bilirubin/albumin molar ratio was smaller upto a molar ratio of 1:1. On the other hand, at any molar ratio higher than 1:1, the increase in bound bilirubin was more significant. Precentage increase in bound bilirubin was similar in all cases on increasing the molar ratio either from

1.0 to 1.5 or from 1.5 to 2.0. This increase became smaller on moving from molar ratio 2.0 to 2.5. The value (% increase in erythrocyte-bound bilirubin) decreased further on increasing the molar ratio from 2.5 to 3.0. This is because at higher molar ratios, binding approached saturation. Further, at bilirubin/albumin molar ratio 2.0, the amount of erythrocyte-bound bilirubin was found four fold with respect to the one at molar ratio 1.0 when bilirubin concentration was 125.5 μM. This was in contrast with the report of Bratlid (1972a) who demonstrated an about eight fold increase in erythrocyte-bound bilirubin on increasing the molar ratio from 1.0 to 2.0 when bilirubin concentration was 160 μM. It should be noted that at bilirubin/albumin molar ratio 2.0, both primary and secondary binding sites on albumin are filled and bound bilirubin at these sites is always in equilibrium with free bilirubin which is available for binding to red blood cells. Binding of bilirubin to red cells is different from that of albumin. Bilirubin acid especially bilirubin acid monoanion is considered to bind to red blood cells (Wennberg, 1988) whereas bilirubin dianion is bound to albumin (Brodersen, 1979). Further, albumin may also bind bilirubin acid monoanion as it can elute the erythrocyte-bound bilirubin. Thus, cellular binding of bilirubin is affected by pH (Bratlid, 1972 b) being higher at low pH values. As our experiments were performed at pH 8.0, small increase in erythrocyte-bound bilirubin at pH 8.0 compared to large increase reported in an earlier study at pH 7.4 can be ascribed to pH effects. pH can affect both

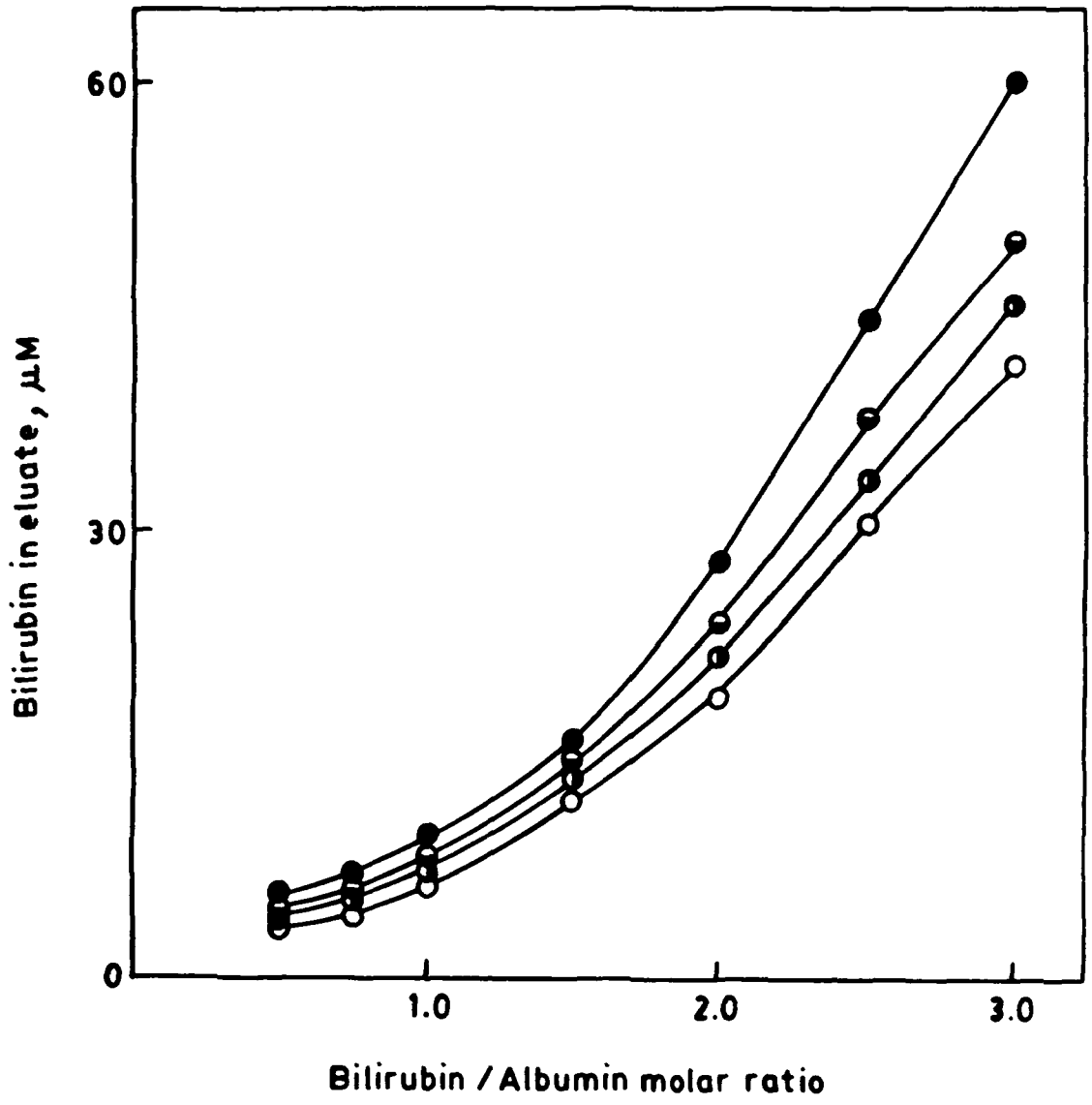

Figure 10. Binding of bilirubin to the erythrocytes of goat (●–●), buffalo (◑–◑), human (◐–◐) and sheep (○–○) at different bilirubin/albumin molar ratios. Albumin concentration was kept constant at 39.5 μM and the bilirubin concentration varied.

the state of bilirubin as well as the physicochemical conditions of bilirubin binding substances on the plasma membrane of red cells as suggested earlier (Sato & Kashiwamata, 1983).

(b) At different bilirubin/albumin molar ratios (keeping the albumin concentration constant while varying the bilirubin concentration)

The binding patterns of bilirubin to the erythrocytes of human, goat, buffalo and sheep at different bilirubin/albumin molar ratios, obtained by keeping albumin concentration constant while varying the bilirubin concentration (Fig. 10) were similar to those obtained at different bilirubin/albumin molar ratios when bilirubin concentration was kept constant and albumin concentration varied. Increase in molar ratio caused increase in erythrocyte-bound bilirubin which was less significant upto a molar ratio 1:1 but became more pronounced above molar ratio 1:1. These results strongly suggest that binding of bilirubin to erythrocytes of all mammalian species studied depends on the concentration of bilirubin and the bilirubin/albumin molar ratio.

(c) At constant bilirubin/albumin molar ratio while varying both the bilirubin and albumin concentrations

At a given constant bilirubin/albumin molar ratio, the binding of bilirubin to erythrocytes increased linearly with increase in bilirubin concentration as shown in Figs. 11 - 14. This finding was consistent with the earlier finding on human erythrocytes (Kaufmann *et al.*, 1967).

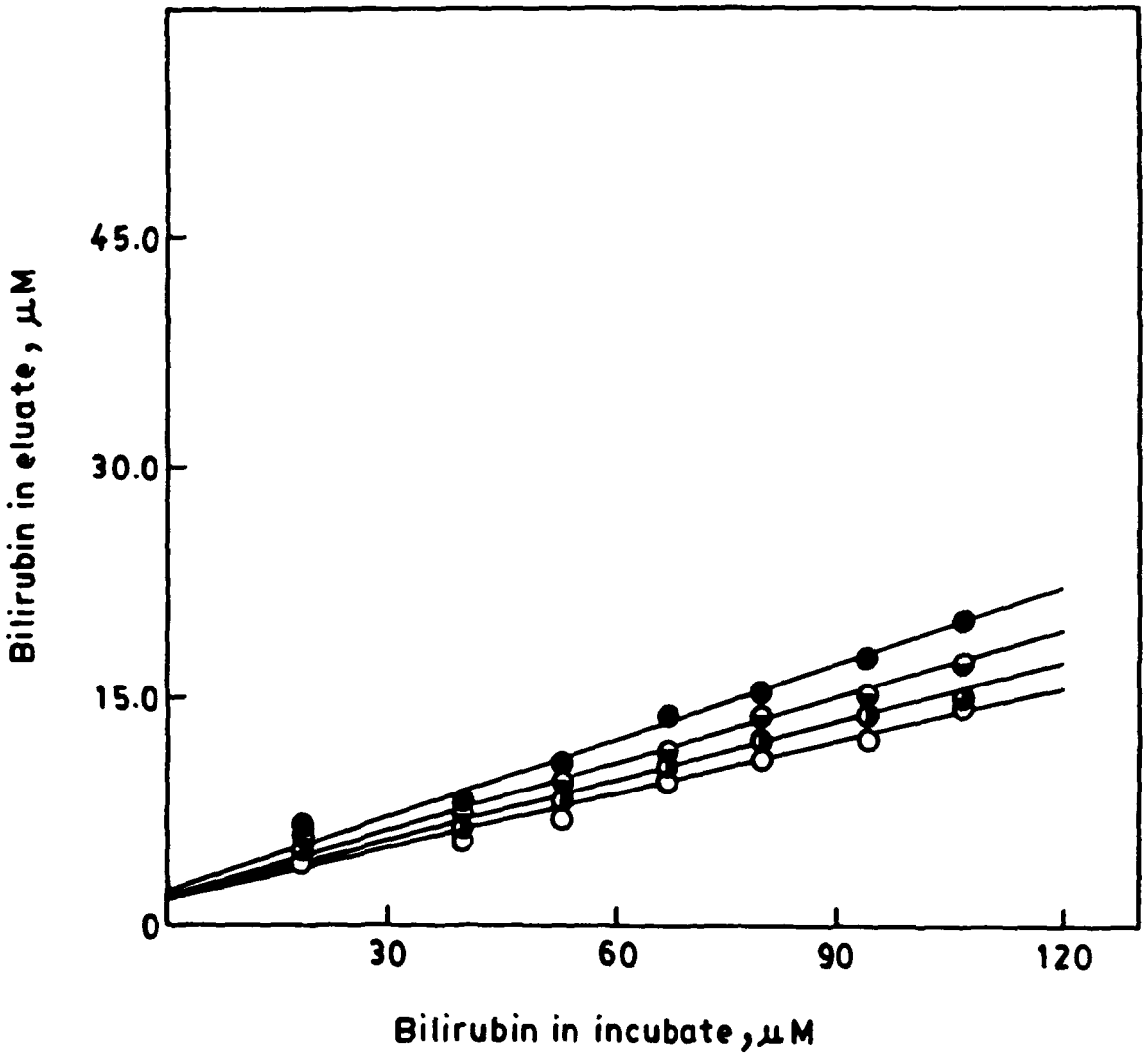

Figure 11. Binding of bilirubin to erythrocytes of goat (●–●) buffalo (θ–θ), human (◑–◑) and sheep (0–0) at constant bilirubin/albumin molar ratio. Both bilirubin and albumin concentrations were varied to obtain a constant bilirubin/albumin molar ratio of 1.5.

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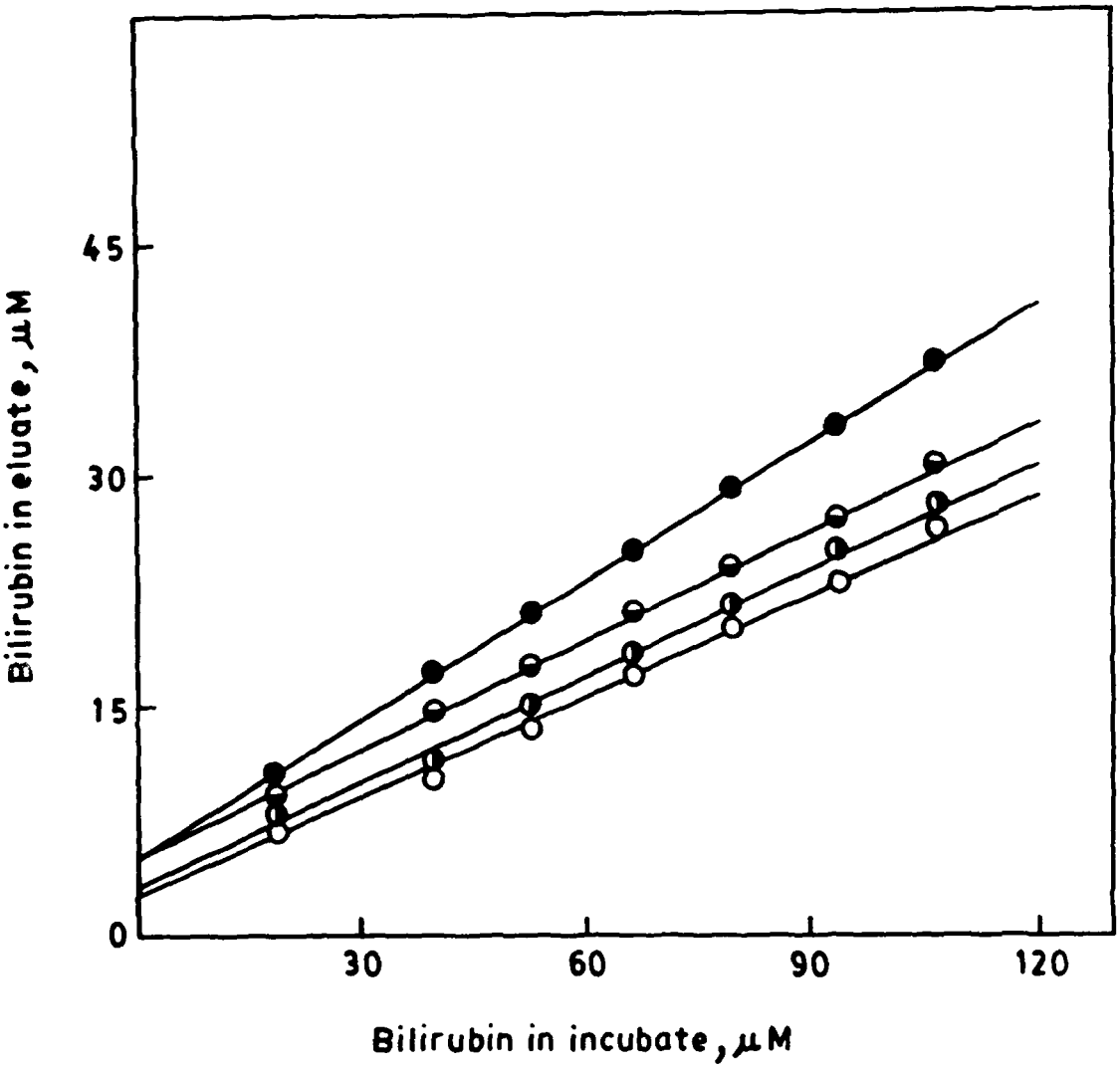

Figure 12. Binding of bilirubin to erythrocytes of goat (●–●) buffalo (0–0), human (●–●) and sheep (0–0) at constant bilirubin/albumin molar ratio. Both bilirubin and albumin concentrations were varied to obtain a constant bilirubin/albumin molar ratio of 2.0.

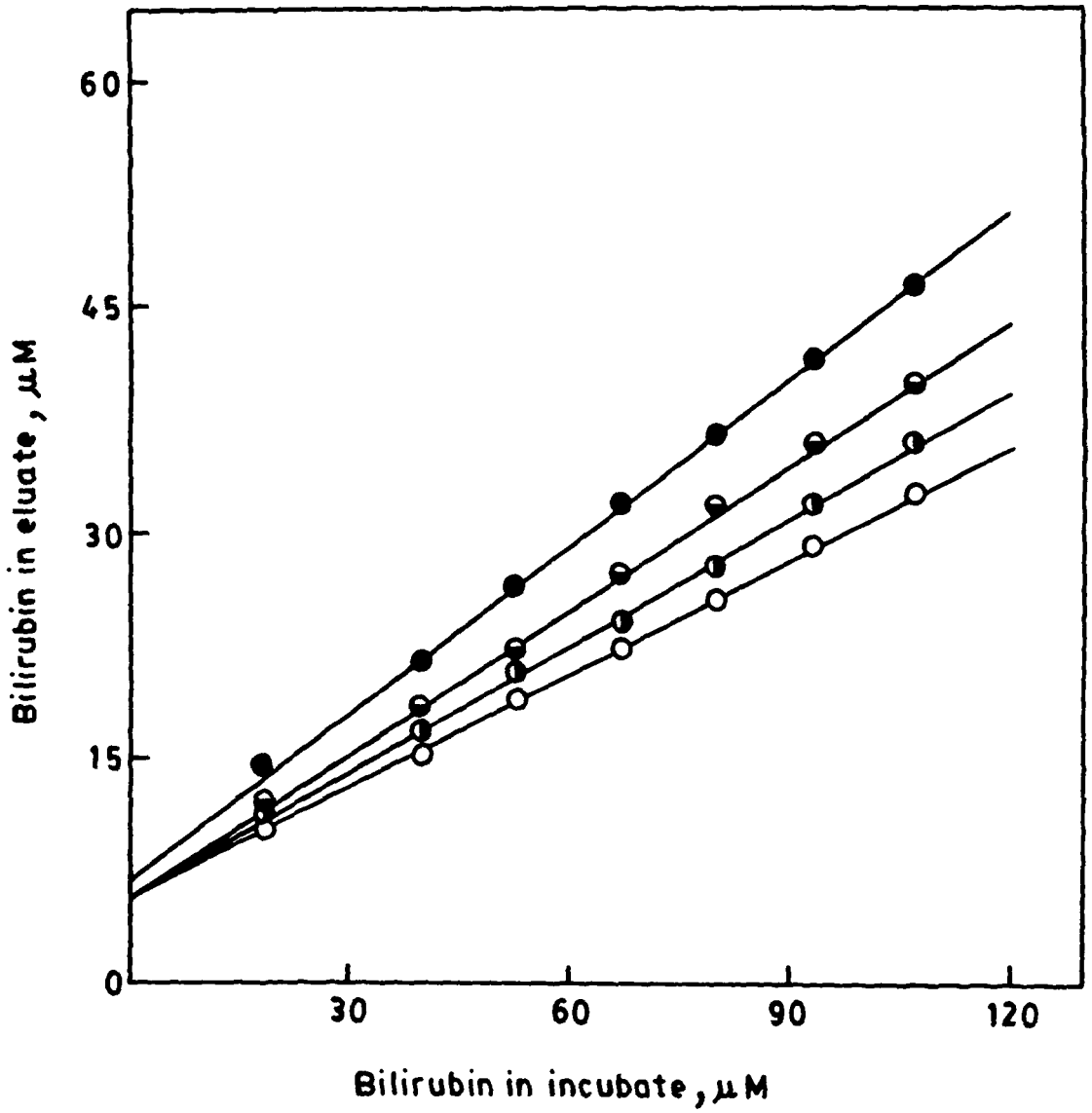

Figure 13. Binding of bilirubin to erythrocytes of goat (●–●) buffalo (◑–◑), human (◐–◐) and sheep (○–○) at constant bilirubin/albumin molar ratio. Both bilirubin and albumin concentrations were varied to obtain a constant bilirubin/albumin molar ratio of 2.5.

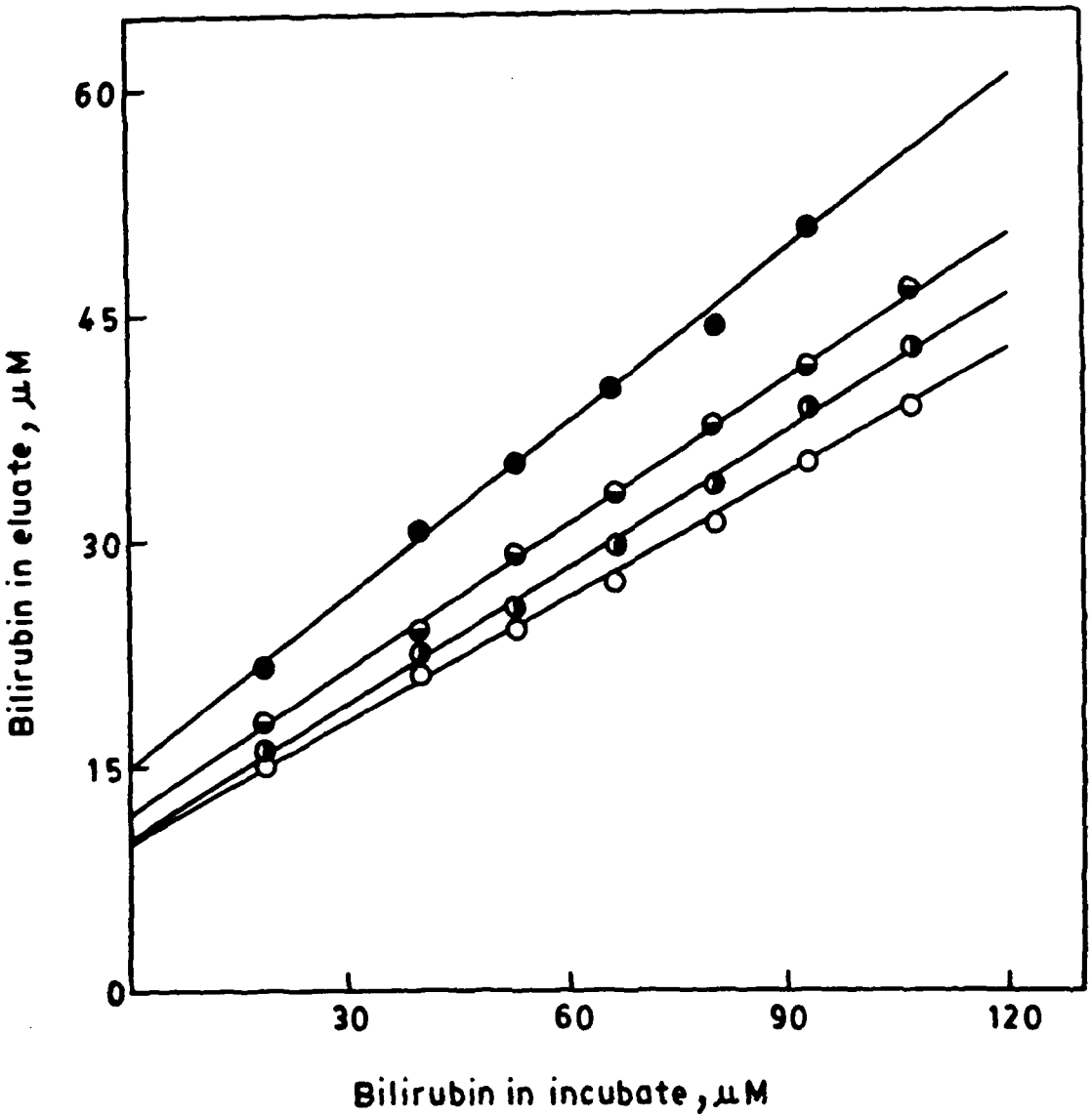

Figure 14. Binding of bilirubin to erythrocytes of goat (●–●) buffalo (θ–θ), human (◑–◑) and sheep (0–0) at constant bilirubin/albumin molar ratio. Both bilirubin and albumin concentrations were varied to obtain a constant bilirubin/albumin molar ratio of 3.0.

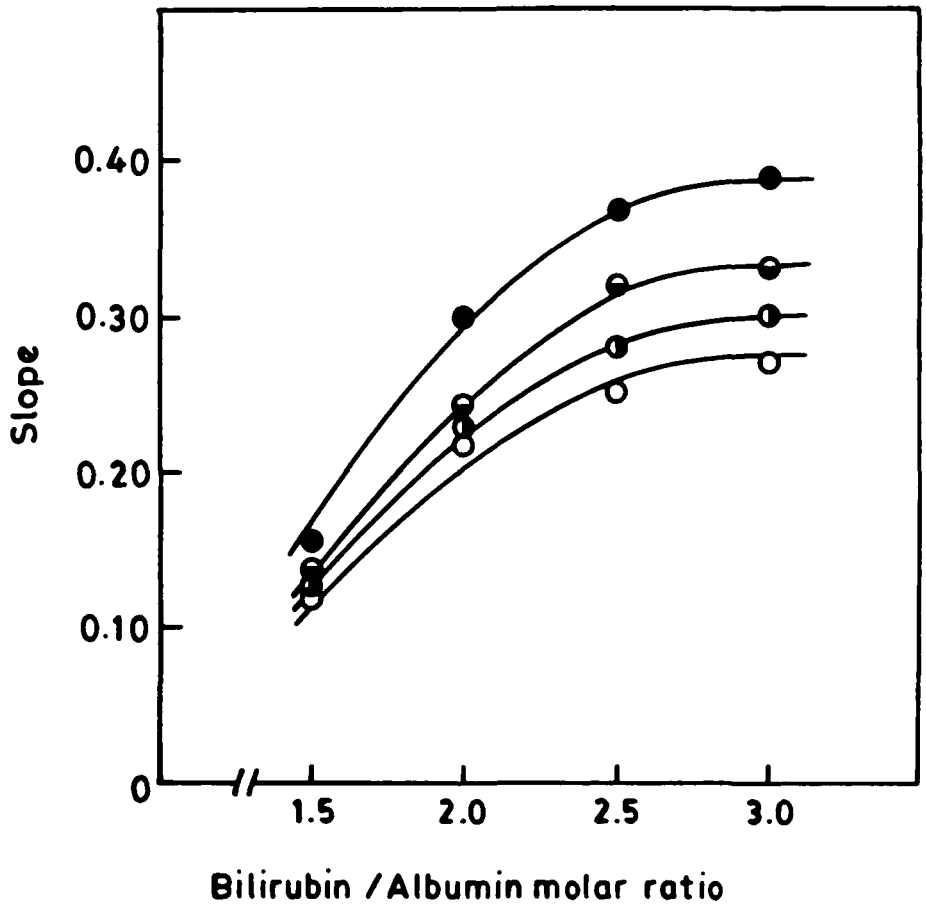

Figure 15. Plots of the slope (taken from Figures 11 -14) versus bilirubin/ albumin molar ratio. Various mammalian species were: goat (●–●), buffalo (0–0), human (0–0) and sheep (0–0).

However, the value of slope of the linear plots at any given bilirubin/ albumin molar ratio was found to be different for different erythrocytes. At any constant bilirubin/albumin molar ratio, the value was highest in case of goat erythrocytes followed by buffalo and human and lowest in case of sheep erythrocytes (see Figs. 11 - 14). Further, with all the species, highest binding was observed at highest molar ratio i.e. 3.0. Thus, the binding was highest in case of goat erythrocytes and lowest in case of sheep erythrocytes.

Values of the slope obtained from these plots were plotted against bilirubin/albumin molar ratio and the results are shown in the Fig. 15. In all cases, initially there was a rapid increase in the value of the slope and then it became somewhat constant at higher molar ratios, i.e. 2.5 and 3.0. In other words, increase in erythrocyte-bound bilirubin per unit increase in bilirubin concentration was found to be dependent upon molar ratio upto a ratio of 2.5 and then became independent above molar ratio 2.5 as the value was nearly constant at molar ratio 2.5 and 3.0. This suggests the saturation of bilirubin binding sites on the erythrocyte membranes at higher molar ratios. From the Fig. 15, it is clear that saturation of bilirubin binding sites is different in different erythrocytes. For example, sheep erythrocytes can be saturated at lower bilirubin concentration as compared to other mammalian erythrocytes.

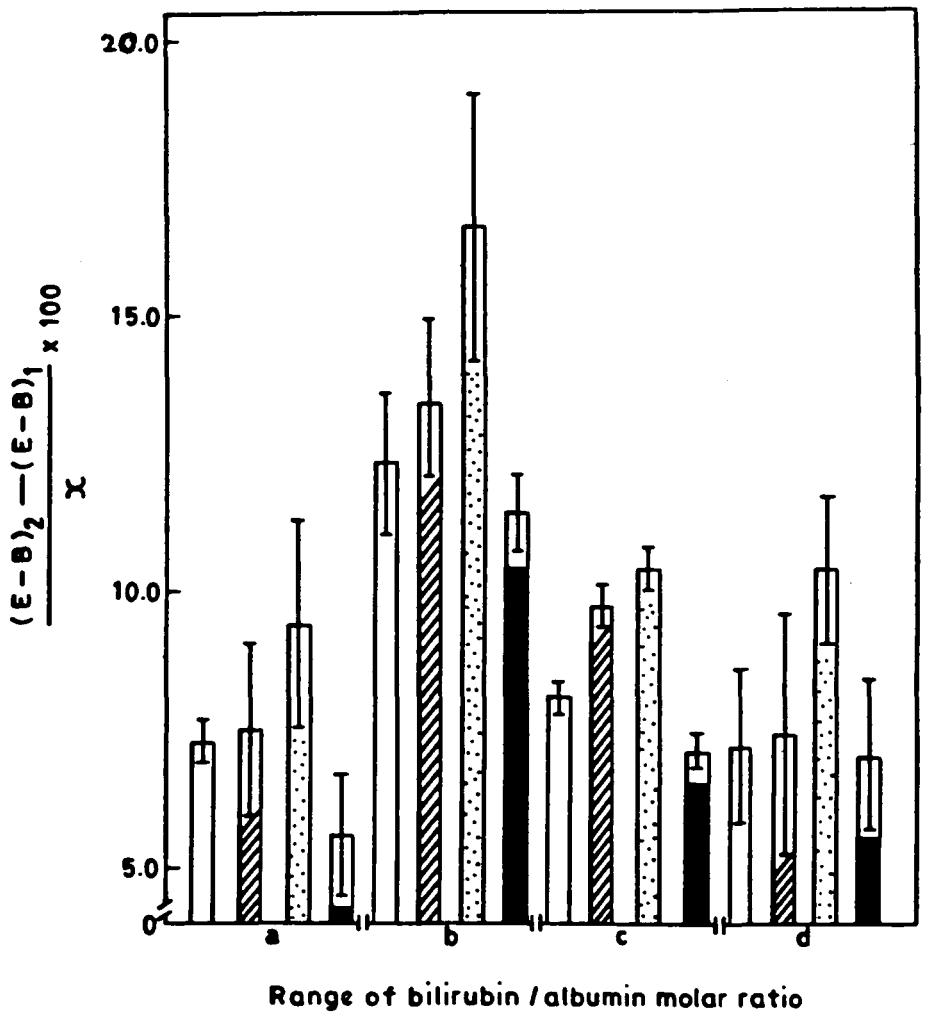

Figure 16. Histogram representing the percentage fractional binding of bilirubin to erythrocytes of various mammalian species i.e. goat (⊡); buffalo (▨); human (□) and sheep (■) at different bilirubin albumin molar ratios. The bilirubin/albumin molar ratios were from (a) 1.0 - 1.5, (b) 1.5 - 2.0, (c) 2.0 - 2.5 and (b) 2.5 - 3.0.

— ***Fractional binding of bilirubin to erythrocytes***

Percentage difference in fractional binding of bilirubin to erythrocytes was calculated using the following equation:

$$\% \text{ difference in fractional bilirubin binding} = \frac{(E\text{-}B)_2\text{-}(E\text{-}B)_1}{X} \times 100$$

where $(E\text{-}B)_2$ and $(E\text{-}B)_1$ are the erythrocyte-bound bilirubin at higher and lower bilirubin/albumin molar ratios respectively and X is the concentration of bilirubin in the incubate ranging from 52.5-125.5 μM. The values, thus obtained, were plotted against the range of bilirubin/ albumin molar ratios and are shown in Fig. 16. As can be seen from the figure, the percentage difference in fractional bilirubin binding to erythrocytes was found highest between bilirubin/albumin molar ratios of 1.5 and 2.0. In other words, greater amount of bilirubin transfers from plasma to cells and the transfer seems to be more pronounced at molar ratios of 1.5 to 2.0. At other molar ratios, percentage difference in fractional bilirubin binding was nearly equal to each other. A comparison of percentage difference in fractional bilirubin binding at molar ratios of 1.5 to 2.0 shows that goat erythrocytes have highest percentage difference, reaching to a value of 16.6 ± 2.4, followed by buffalo (13.4 ± 1.9) and human erythrocytes (12.3 ± 3.0) whereas sheep erythrocytes have lowest percentage difference, reaching to a value of 11.4 ± 0.7. These data suggest that number of bilirubin binding sites in goat

erythrocytes is maximum followed by buffalo and human erythrocytes and minimum in sheep erythrocytes.

— ***Relative percentage difference in bound bilirubin by the erythrocytes of sheep, buffalo and goat with respect to human erythrocytes***

The relative percentage difference in the bilirubin bound to erythrocytes of sheep, buffalo and goat with respect to bilirubin bound to human erythrocytes was calculated using the following equation:

$$\text{Relative \% difference in bound bilirubin} = \frac{(E\text{-}B)_x - (E\text{-}B)_H}{(E\text{-}B)_H} \times 100$$

where $(E\text{-}B)_x$ is the concentration of bilirubin bound to goat, sheep or buffalo erythrocytes, $(E\text{-}B)_H$ is the concentration of bilirubin bound to human erythrocytes. The values obtained at different bilirubin/albumin molar ratios in several experiments are given in Table II. At all molar ratios above 1.0, the value of relative percentage difference in erythrocyte-bound bilirubin with respect to bilirubin bound to human erythrocytes was found to be more or less similar. A comparison of the data shows that goat and buffalo erythrocytes bind bilirubin 31.8% and 11.4% respectively more as compared to human erythrocytes. On the other hand, sheep erythrocytes bind 8.7% less bilirubin as compared to human erythrocytes. Thus, binding of bilirubin was maximum with goat erythrocytes and minimum with sheep erythrocytes.

TABLE - II

Relative percentagedifference in bilirubin binding by erythrocytes of different species with respect to human erythrocytes in presence of respective plasma albumins

Bilirubin/albumin molar ratio	Relative % difference in bound bilirubin by		
	Sheep erythrocytes (%)	Buffalo erythrocytes (%)	Goat erythrocytes (%)
1.5	-10.6±3.0	12.5±2.7	30.1±3.6
2.0	- 8.7±2.1	11.0±2.1	32.7±2.5
2.5	- 8.5±1.4	12.2±2.9	30.0±2.3
3.0	- 7.4±1.2	9.8±1.3	34.5±2.2
Mean	- 8.7±1.9	11.4±2.3	31.8±2.7

* Each value represents a mean of 13 observations.

— ***Effect of plasma albumin on the binding of bilirubin to erythrocytes***

Although it is reported that charcoal-treated serum albumins from different species i.e. human, bovine, rabbit and chicken have different affinities for bilirubin (Blauer *et al.*,1967),however, we did not find any difference in the affinities of human, goat, buffalo and sheep plasma albumins for bilirubin. This is based on the finding that elution of erythrocyte-bound bilirubin with human, buffalo, goat and sheep plasma albumins yielded the same amount of bilirubin from human erythrocytes (see Table III). Further, in all the mammalian species studied, the binding of bilirubin to erythrocytes was found to be more or less completely protected in the presence of their respective albumins at a bilirubin/ albumin molar ratio of 0.5 as shown in Fig. 17 as well as in Table IV, in which the ratio of erythrocyte-bound bilirubin in the presence of albumin (v) to erythrocyte-bound bilirubin in the absence of albumin (v_o) was plotted against bilirubin/albumin molar ratio (M.R.). Due to the similar protection of binding of bilirubin to erythrocytes at bilirubin/ albumin molar ratio of 0.5 in all the mammalian species studied, we can reasonably claim that the difference in bilirubin bound to erythrocytes might be due to the difference in the affinities of receptors or due to difference in the number of receptors or due to difference in both the affinities and number of receptors present on these erythrocytes. The binding seems to be dependent upon the amount of available free

TABLE III

Comparison of elution behaviour of bilirubin bound to human erythrocytes by different concentration of different plasma albumins, namely, human, goat, buffalo and sheep plasma

Concentration of plasma albumin used for elution	Concentration of erythrocyte-bound bilirubin eluted by			
(mg/ml)	HPA (μM)	BuPA (μM)	GPA (μM)	SPA (μM)
1.0	24	20.8	25.6	27.2
5.0	57.6	52.8	60.8	60.8
10.0	75.2	76.8	76.8	75.2
15.0	80.0	81.6	80.0	78.4
20.0	88.0	91.2	89.6	91.2
25.0	94.4	86.0	94.4	94.4
30.0	96.0	96.0	94.4	94.4

HPA - Human plasma albumin BuPA - Buffalo plasma albumin

GPA - Goat plasma albumin SPA - Sheep plasma albumin

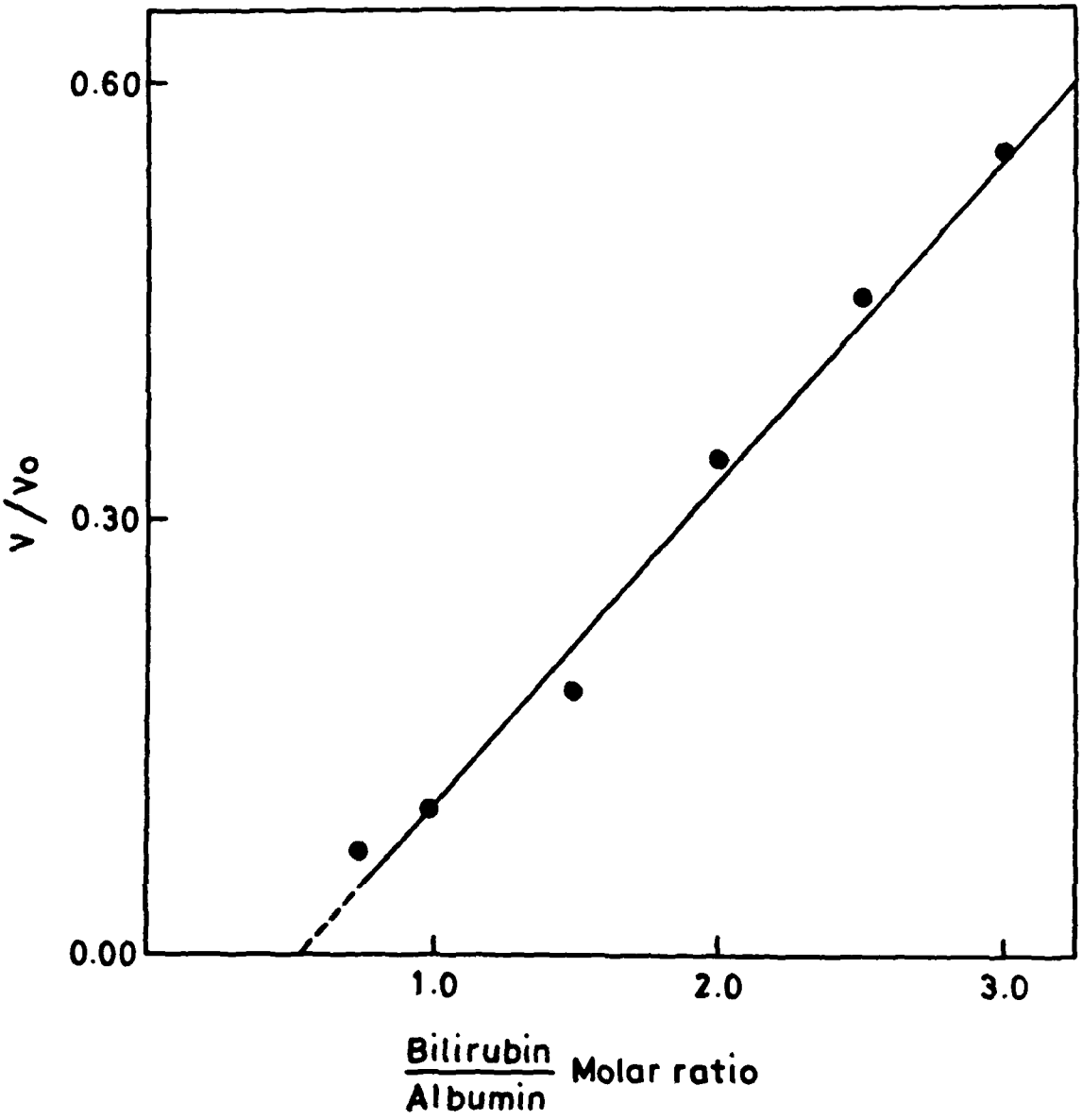

Figure 17. Plots of the ratio of bilirubin bound to human erythrocytes in presence as well as in absence of albumin against the bilirubin/ albumin molar ratio.

TABLE IV

Effect of plasma albumin on the erythrocyte-bound bilirubin

System (erythrocytes/albumin)	m	c	M.R.*
Human	0.22	- 0.12	0.53
Buffalo	0.23	- 0.12	0.53
Goat	0.28	- 0.16	0.55
Sheep	0.21	- 0.11	0.53

Values of slope (m) and intercept (c) were taken from the following equation

v/v_o = m X Bilirubin/albumin molar ratio (M.R.) + C

where v is the erythrocyte-bound bilirubin in presence of albumin and v_o is the erythrocyte-bound bilirubin in absence of albumin.

M.R.* intercept on X- axis.

unconjugated bilirubin. Albumin does not compete with the binding of bilirubin to erythrocytes but lowers the amount of free unconjugated bilirubin which is available to erythrocytes.

(d) Binding of bilirubin to erythrocytes in the absence of albumin

Binding of bilirubin to erythrocytes of human, goat buffalo and sheep was studied in the absence of albumin by incubating the cells with increasing concentrations of bilirubin. The results of the erythrocyte-bound bilirubin and the bilirubin in the incubate are shown in Fig. 18. In all the cases, curves showed a Michaelian saturation. These are similar to the one obtained earlier using human erythrocytes (Hayer *et al.*, 1989). From the Fig. 18, it can be seen that human, goat, buffalo and sheep erythrocytes have different saturation capacities. For the determination of dissociation constants of the bilirubin-receptor complex and saturation, the data from Fig. 18 were transformed into double reciprocal plots and the values of the slope and intercept were used for the calculation of the dissociation constant of the bilirubin-receptor complex and saturation. For simplicity, the double reciprocal plot obtained with human erythrocytes is shown in Fig. 19. The values of the dissociation constant of the bilirubin-receptor complex and saturation are listed in Table V. In the case of human erythrocytes, saturation corresponded to a binding of 76.9 μmoles of bilirubin per 1000 ml erythrocytes and the dissociation constant of bilirubin-receptor complex

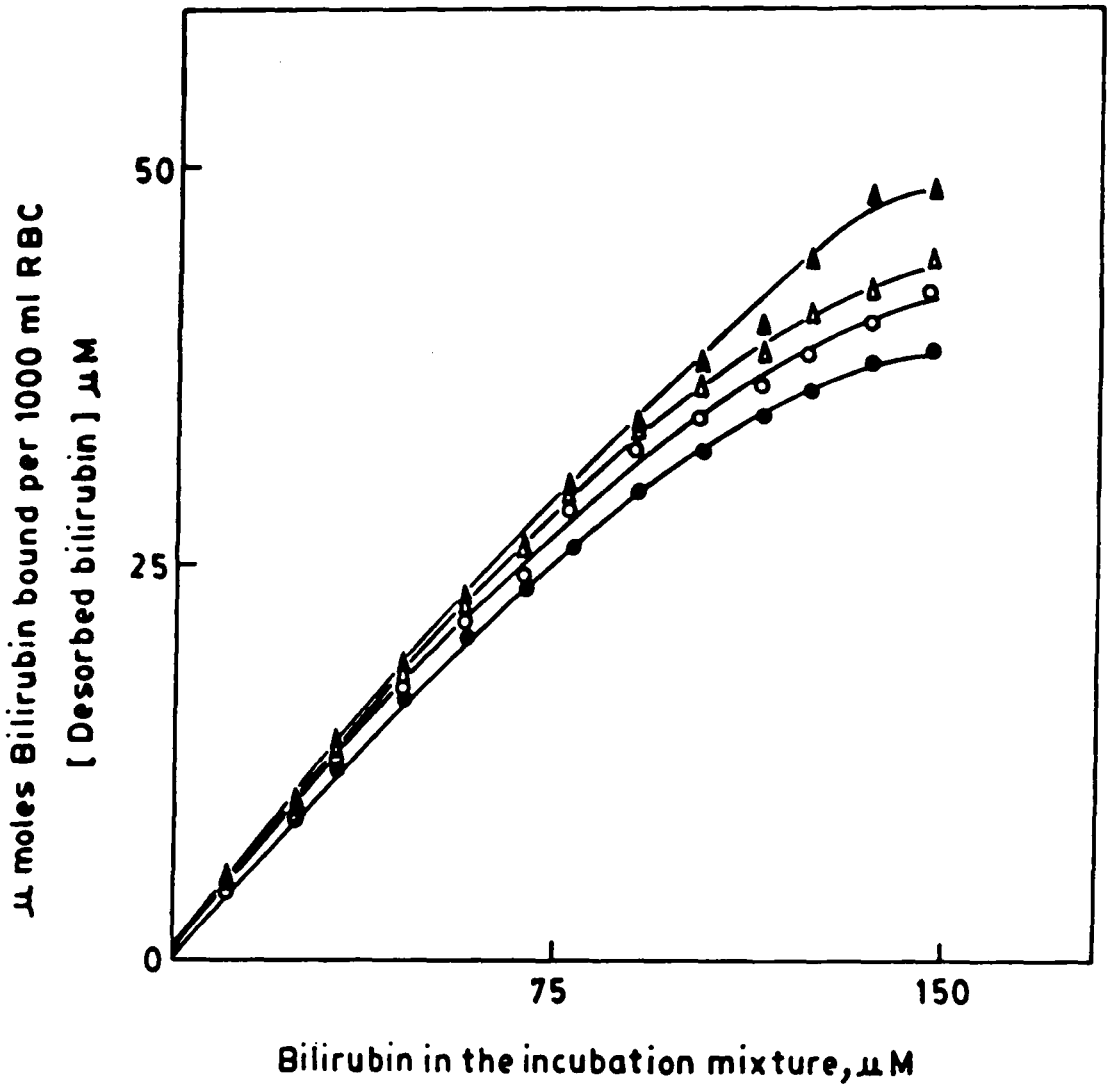

Figure 18. Binding of bilirubin by the erythrocytes of goat (▲–▲), buffalo (Δ–Δ), human (0–0) and sheep (●–●) in the absence of plasma albumin.

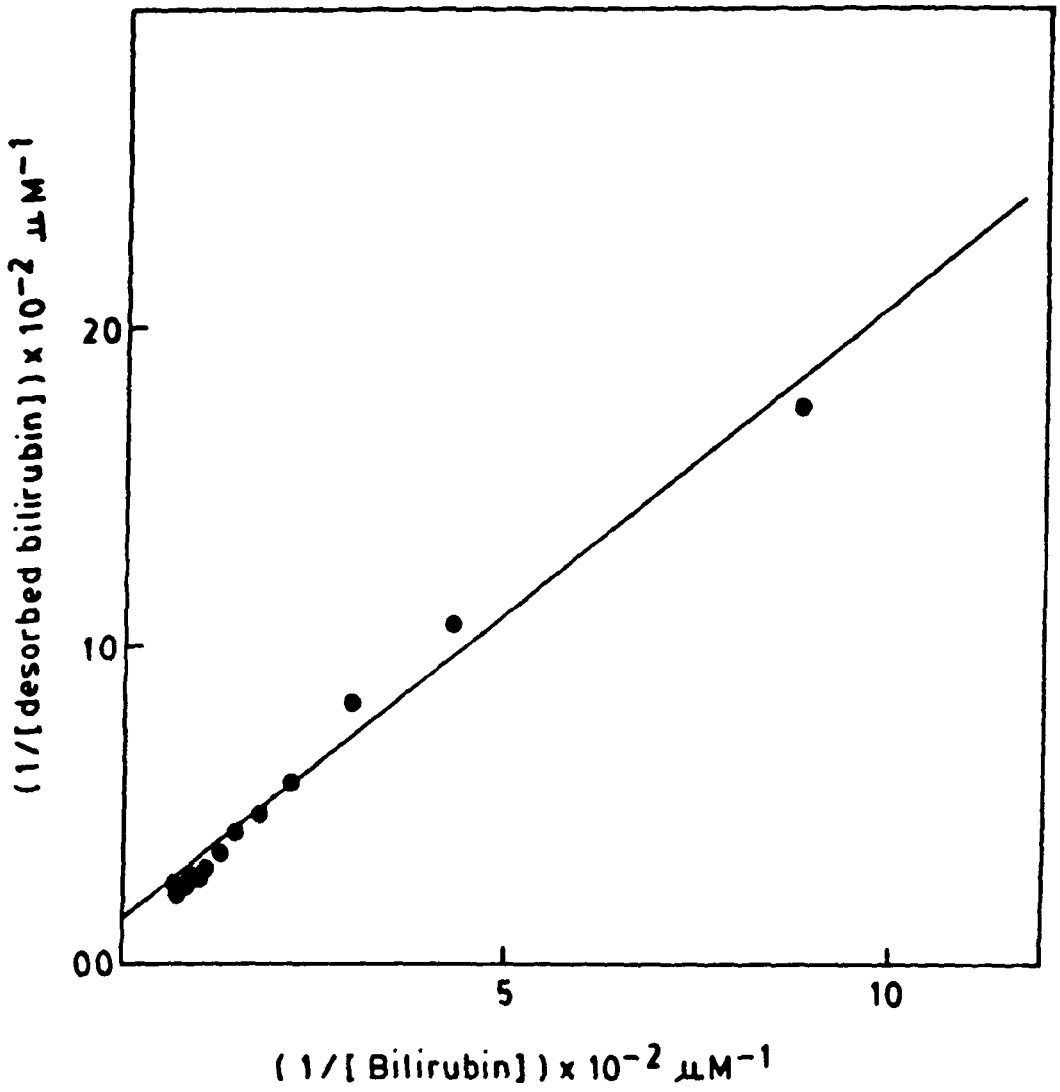

Figure 19. Double reciprocal plot between inverse of erythrocyte-bound bilirubin concentration and inverse of bilirubin concentration in the incubate for human erythrocytes.

TABLE V

Interaction parameters of bilirubin with erythrocytes of different species.

Species	Saturation μ moles of bilirubin/ 1000 ml RBC	Dissociation constant (μmol/l)
Human	76.9	150.0
Buffalo	83.3	155.8
Goat	125.9	265.7
Sheep	62.5	115.6

was found to be $150x10^{-6}$ moles per litre. This value of the dissociation constant was 0.9 times lesser than the one reported earlier (Hayer *et al.*, 1989). A comparison of dissociation constant of the bilirubin-receptor complex ($150x10^{-6}$ moles/litre) with that of the bilirubin-HSA complex ($1.5x10^{-8}$ moles/litre) for primary binding site (Brodersen, 1979) suggests that the binding of unconjugated bilirubin to erythrocytes, even for high bilirubinemia (150 moles/litre) is extremely weak. From the Table V, it can be seen that goat erythrocytes have the highest dissociation constant whereas sheep erythrocytes have the lowest. Buffalo erythrocytes bind bilirubin with a dissociation constant similar to human erythrocytes. Similarly saturable binding on the erythrocyte membranes was maximum with goat erythrocytes whereas sheep erythrocytes bound the lowest amount of bilirubin. Thus, it appears that the number of saturable bilirubin binding sites on goat erythrocytes is highest of all the species studied whereas the number of binding sites on sheep erythrocytes is the lowest. Human and buffalo erythrocytes have the same number of binding sites and are in between goat and sheep erythrocytes. In contrast, the affinity of these sites was highest in sheep erythrocytes and lowest in goat erythrocytes. Human and buffalo erythrocytes have similar affinities which are in between the two. The finding of the differences in the binding of bilirubin to erythrocytes in the absence of albumin also explained the previous finding that the binding differences of bilirubin in goat, sheep, buffalo and human erythrocytes in presence of albumin

were due to the differences in the number of saturable binding sites as well as the bilirubin binding affinities of erythrocytes of these species. Since the membrane make-up of proteins (Lenard, 1970) and lipids (Barenholz & Thompson, 1980) and other properties such as thermal behaviour (Calhoun & Shipley, 1979), function of membrane proteins (Kramer *et al.,* 1972), stability to osmotic shock, permeability to molecules for which no specific transport system exists (Deuticke, 1977) is different in these erythrocytes, this might account for the differences in the affinity as well as in the number of saturable binding sites on these erythrocytes. The results on the binding studies of bilirubin to goat, human, buffalo and sheep erythrocytes suggest that bilirubin receptors on the membrane of different erythrocytes differ not only in the number, but also in their receptor affinities. These differences in affinity may be due to the different structures of these receptors or might be due to other factors.

REFERENCE

Barenholz, Y. and Thompson, T.E. (1980) Biochim. Biophys. Acta **604**, 129-158

Barnhart, J.L. and Clarenburg, R. (1973) Pro. Soc. Exp. Biol. Med. **142**, 1101-1103

Beaven, G.H., d'Albis, A. and Gratzer, W.B., (1973) Eur. J. Biochem. **33**, 500-510

Berde, C.B., Hudson, B.S., Simoni, R.D. and Sklar, L.A. (1979) J. Biol. Chem. **254**, 391-400

Blanckaert, N., Compernolle, F., Heirwegh, K.P.M., DeGroote, J, and Fevery, J. (1974) Digestion **10**, 315

Blanckaert, N., Fevery, J., Compernolle, F. and Hairwegh, K.P.H. (1975) Gastroenterology **69**, A-10/810

Blanckaert, N., Heirwegh, K.P.M. and Compernolle , F. (1976) Biochem. J. **155**, 405-417

Blauer, G., Harmatz, D. and Snir, J. (1972) Biochem. Biophys. Acta **278**, 68-88

Blauer, G., Lavie, E. and Silfen, J. (1977) Biochimica et Biophysica Acta **492**, 64-69

Blumenthal, S.G., Taggart, D.B., Ikeda, R.M., Ruebner, B. and Bergstrom, D.E. (1977) Biochem. J. **167**, 535

Bonnett, R., Davies, J.E. and Hursthouse M.B. (1976) Nature **262**, 327-328

Bouillerot, A., Fessard, C. and Sauger, F. (1981) Clin. Chem. Acta **109**, 39-44

Bratlid, D. (1972a) Scand. J.Clin. Lab. Invest. **29**, 91-97

Bratlid, D. (1972b) Scand, J.Clin. Lab. Invest. **29**, 453-459

Bratlid, D. and Rugstad, H.E. (1972) Scand. J.Clin. Lab. Invest. **29**, 461-466

Brodersen, R. (1974). J. Clin. Invest. **54**, 1353-1364

Brodersen, R. (1979) Crit. Rev. Clin. Lab. Sci. **11**, 305-399

Brodersen, R. (1979) J. Biol. Chem. **254**, 2364-2369

Brodersen, R (1980) J. Pediatr. **96**, 349-356

Brodersen, R. and Funding, L. (1977) Scand. J. Clin. Lab. Invest. **37**, 257-266

Brodersen, R., Sjo"din, T. and Sjo"holm, I. (1977) J. Biol. Chem.**252**, 5067-5072

Brown, S. (1976) Biochem. J. **159**, 23

Byers, R.K., Paine, R.S. and Crothers, B. (1955) Pediatrics **15**, 248-254

Calhoun, W.I. and Shipley, G.G. (1979) Biochim. Biophys. Acta **555**, 436-441

Carey, M.C. and Korestsky, A.D. (1979) Biochem J. **179**, 675

Chen, R.F. (1973) The fluorescence of bilirubin-albumin complexs, in Cell Biology, Thaer, A.A. and Sernetz, M., Eds, Springer, Berlin. 239-248

Cheung, W.H., Sawitsky, A. and Isenberg, H.D. (1966) transfusion **6**, 475-486

Compernolle, F., vanHees, G.P., Fevery, J., and Heirwegh, K.P.M. (1971) Biochem. J. **125**, 811

Constantopoulos. A. and Mutsaniotis, N. (1976) Cytobios **17**, 17-120

Davis, B.J. (1964) Ann., N.Y. Acad. Sci. **121**, 404-427

Deuticke, B. (1977) Rev. Physiol. Biochem. Pharm. **78**, 1-97

Evans, W.E., Rodman, J.H., Relling, M.V., Petros, W.P., Stewart, C.F., Pui, C.H. and Rivera, G.K. (1992) J.Pharmacol Exp. Ther. **260**, 71-77

Fehske, K.J., Muller, W.E. and wollert, W. (1979) Biochem. Biophys. Acta **577**, 346-359

Fenwick, J.D. (1975) J. Laryngol. Otol. **89**, 925-932

Fevery, J., Vijver, M.V.D., Michiels, R. and Heirwegh, K.P.M. (1977) Biochem. J. **164**, 737

Fog, J. (1958) Scand. J. Clin. Lab. Invest. **10**, 241-245

Folin, O. and Ciocalteu, V. (1927) J. Biol. Chem. **73**, 627-650

Gray, R.D. and Stroupe, S.D. (1978) J. Biol. Chem. **253**, 4370-4377

Gulian, J.M. (1987) J.Antimicrobial Chemotherapy **19**, 823-829

Hansen, T.W.R. and Bratlid, D. (1986) Acta Paediatr Scand **75**, 513-522

Hayer, M., Piva, M-T., Sieso, V. and de Bornier, B.M. (1989) Clin. Chem. Acta **186**, 345-350

Hailmeyer, L. (1931) Biochem. Z. **232**, 229.

Heirwegh, K.P.M., Blanckaert, N., Compernolle, F., Fevery, J. and Zaman, Z. (1977) Biochem. Soc. Trans. **5**, 316

Hervieux, J. De l'ictere des nouveau. These. Med. Paris (1847)

Hyman, C.B., Keaster, J. and Hanson, V. (1969) Am. J.Dis. Child **117**, 395-405

Jacobsen, J. (1969) FEBS Lett. **5**, 112-114

Jacobsen, J. (1977) Int. J. Pept. Prot. Res. **9**, 235-239

Jacobsen, J. and Brodersen, R. (1976) Birth Defects: Orig. Artic. Ser. **12**, 175-178

Jacobsen, J. and Brodersen, R. (1983) J.Biol. Chem. **258**, 6319-6326

Jacobsen, J. and Wennberg, R.P. (1974) Clin. Chem. **20**, 783-789

Jendrassik, L. and Grof, P. (1938) Biochem. Ztschr. **297**, 81-89

Kapool, C.L. (1975) Curr. Sci. **44**, 82-84

Karp. W. (1979) Pediatrics **64**, 361-368

Kaufmann, N.A., Kapitulnik, J. and Blondheim, S.H. (1969) Pediatrics **44**, 543-

Kaufmann, N.A., Simcha, A.J. and Blondheim, S.H.(1967) Clin. Sci. **33**, 201-208

Keaster, J., Hyman, C.B. and Harris, I. (1969) Am. J. Dis. Child **117**, 406-410

Knobloch, E. and Miler, I. (1986) Folia Microbiologica **31**, 387-393

Kramer, R., Schlatter, C. and Zahler, P. (1972) Biochim. Biophys. Acta **282**, 146-156

Krasner, J., Giacoia, G.P. and Yaffe, S.J. (1973) Ann. N.Y. Acad. Sci., **226**, 101-114

Kucerrova, L. and Jirsa, M. (1969) Sb. Lek. **71**, 114-118

Lamola, A.A., Eisinger, J., Blumberg, W.E., Pathel, S.C. and Flores, J. (1979) Analyl. Biochem. **100**, 25-42

Lenard, J. (1970) Biochemistry **9**, 5037-5040

Levine, R.L. (1977) Clin. Chem. **23**, 2292-2301

Levine, R.L., Fredricks, W.R. and Rapport, S.I. (1982) Pediatries **69**, 255-259

Lie, S.O. and Bratlid, D. (1970) Scand. J. Clin. Lab. Invest. **26**, 37-41

Lightner, D.A. and Park, Y. (1977) J. Heterocycl. Chem. **14**, 415-422

Lowry, O.H., Rosebrough, N.J., Forr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265-275

Maisels, M.J. (1972) Pediatr. Clin. North Am. **19**, 447-501

Martin N.H. (1949) J. Am. Chem. Soc. **71**, 1230-1235

McDonagh, A.F. (1979) The Porphyrins, Vol.6, Chap 6. Dolphin, D., Ed., Academic Press

Metze, H. (1977) Klinische Padiatrie **189**, 393-400

Miler, I., Vetvicka, V., Sima, P. and Ta'borsky', L. (1985) Folia Microbiologica **30**, 267-271

Moiny, G., Thirion, A. and Deby, C. (1990) Thrombosis research **59**, 413-416

Mustafa, M.G. and King T.E. (1970) J.Biol. Chem. **245**, 1084-1089

Nagaoka, S. and Cowger, M.L. (1978) J. Biol. Chem. **253**, 2005-2011

Nelson, Th., Jacobsen, J. and Wennberg, R.P. (1974) Pediatr. Res. **8**, 963-967

Odell, G.B. (1959) J. Pediat. **55**, 268-279

Odell, G.B. (1959) J. Clin. Invest. **38**, 823-833

Odell, G.B. (1965) Proc. Soc. Exp. Biol. **120**, 352-354

Odell, G.B. (1966) J.Pediatries **68**, 164-180

Odell, G.B. (1970) *Pediatrics* **46**, 16-24

Odell, G.B. (1980) Neonatal hyperbilirubinaemia. Grune and Stratton, New York.

Odell, G.B. Story, G.N.B. and Rosenberg, L.A. (1970) J.Pediatr. **76**, 12-21

Onks, D.L., Harris, J.F. and Robertson, A.F. (1991) Pharmacol. Toxicol. **68**, 328-331

Oski, F.A. and Naiman, J.L. (1963) J. Pediatr. **63**, 1034-1037

Ostrow, J.D. and Schmid, R. (1963) J. Clin. Invest. **42**, 1286-

Overbeek, J.T.G., Vink, C.L.J. and Deenstra, H. (1955) Recl. Trav. Chim. Pays-Bas **74**, 81

Pedersen, A.O., Schonheyder, F. and Brodersen, R. (1977) Eur. J. Biochem. **72**, 213-221

Perlstein, M.A. (1960) Pedicatr. Clin. M. Am. **7**, 665-687

Peters, T., Jr. (1970) Adv. Din. Chem. **13**, 37-111

Peters, T., Jr (1975). In" The Plasma Proteins" (F.W. Putnam.) Vol. I. pp. 133-181, Academic Press, New York

Peters, T. Jr. (1980) "serum Albumin An Overview and Bibliography" Miles Laboratories, Elkhart, Indiana.

Petres, T., Jr. (1985). Adv. Protein Chem. **37**, 161-245

Rasmussen, L.F. and Wennberg, R.P. (1972) Res. Commun. Chem. Pathol. Pharmacol. **3**, 567-578

Reed, R.G., Feldhoff, R.C., Clute, O.L. and Peters, T. (1975) Biochemistry **14**, 4578-4583

Robertson, A., Karp, W. and Brodersen, R. (1991) Acta Paediatr. scand. **80**, 1119-1127

Rosenstein, B.S., Duecre, J.M. and Cummings, S.W. (1983) Mutat Res. **112**, 397-406

Rosenstein, B.S. and Ducore, J.M. (1984) Pediatr. Res. **18**, 3-6

Rubalteli, F.F. and Jori, G. (1979) Photochem. Photobiol. **29**, 991-

Sato, H., Aono, S., Semba, R. and Kashiwamata, S. (1987) Biochem. J. **248**, 21-26

Sato, H. and Kashiwamata, S. (1983) Biochem. J. **210**, 489-496

Schettini, F. and Meloni, T. (1964) Acta haemat. (Basel) **32**, 35

Schmid, R., Diamond, I., Hammaker, L. and Gundersen, C.B. (1965) Nature **206**, 1041-1043

Schmid, R., McDonough, A.F. (1978) Formation and metabolism of bile pigments in vivo. In: The Porphyrins. Dolphin D. Academic Press

Schmorl, G. (1904) Verh. Dtsch. Pathol. Ges. **6**, 109-115

Tayyab, S. and Qasim, M.A. (1988) Med. Sci. Res. **16**, 893-895

Tayyab, S. and Qasim, M.A. (1990) Biochem. Int. **20**, 405-415

Thaler, M.M. (1971) Nature New Biol. **230**, 218-219

Thaler, M.M. and Wennberg, R.P. (1977) Pediatr. Res. **11**, 167-171

Watson, D. (1962) Clin. Chem. Acta. **7**, 733-734

Watson, D. (1962) Clin. Sci. **22**,435-445

Wennberg, R.P. (1971) Albumin binding of bilirubin, Theorelical, experimental and clinical consideration (Abst). Procd. XIII. Int. Congr. Pediatrics, Vol. 1. 311, Vienna.

Wennberg, R.P. (1988) Pediatric Res. **23**, 443-447

Wennberg, R.P., Ahlfors, C.F., and Rasmussen, L.F. (1979) Early Hum. Dev. **3**, 353-372

Wennberg, R.P. and Rasmussen, L.P. (1978) Pediatric Res. **12**, 536

Yamaji, K. (1975) Chem. Abstr. **83**, 232

Zemet, P. and Chunga, F. (1971) Acta Paediatr. Scand. **60**, 33-35

Zetterstrom, R. and Ernster, L. (1956) Nature **178**, 1335-1337

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LIST OF PUBLICATIONS / PRESENTATIONS

1. "Interference of sodium azide with the quantitation of bilirubin: modification of Fog's method to eliminate azide interference".

   Tayyab, S. and **Ali, M.K.**

   Analytical Biochemistry (1995) **224,** 542-546.

2. "Alkaline azobilirubin color reaction to determine sodium azide"

   Tayyab, S. and **Ali, M.K.**

   Clinical Chemistry (1995) **41,** 764.

3. "Binding of bilirubin to erythrocytes from different mammalian species"

   Tayyab, S. and **Ali, M.K.**

   Comparative Biochemistry and Physiology (1995) in press.

4. "Bilirubin-red blood cells interaction: an approach towards understanding the mechanism of bilirubin encephalopathy"

   Ali, M.K. and Tayyab, S.

   Proceedings of 16th International Congress of Biochemistry and Molecular Biology, held at New Delhi, India, from september 19-22, 1994, **V-II** Abs. No. P 11-49.